WHAT PREVENTS HYBRIDISATION IN *CELMISIA*?

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“*A most beautiful genus, abundant in New Zealand, and, as in all the other large genera of these islands, the species are very variable, difficult to distinguish, and intermediate forms may be expected...*”

– J.D. Hooker 1864

Abstract

Hybrids are common, being found in about 25% of all plant species, but the isolating barriers which preserve species integrity are poorly studied. I investigated this question in the large New Zealand genus *Celmisia* Cass. (Asteraceae), which hybridises readily in cultivation, but wild hybrids are relatively rare. My study quantitatively tests four potential reproductive isolating barriers in 12 sympatric species of *Celmisia* found in the Craigieburn Range, inland Canterbury, New Zealand. I examined two potential prezygotic reproductive isolating barriers (flowering phenology and pollinator specialisation), and two potential postzygotic barriers (pre-dispersal seed predation and hybrid seed germination). I used null models to test whether *Celmisia* species had temporally segregated flowering times, and found that some *Celmisia* are temporally segregated and thus less likely to form hybrids. I used experimental pair-wise flowering arrays to observe insect visitation to six different *Celmisia* species pairs. While I found no difference in the overall pollinator community, several insect families showed preferences for some *Celmisia* species. Furthermore, I found that subtle floral character differences were driving these insect preferences. In particular, I found scape height to be positively associated with insect visitation with taller *Celmisia* being favoured over shorter species. Insect preferences did not translate into strong floral constancy, therefore indicating that *Celmisia* flower visitors are likely to be a weak barrier to hybridisation. I reared a range of insect seed predators from field-collected capitula of the hybrid *C. x pseudolyallii* and both parent species (*C. lyallii* and *C. spectabilis*). There was no overall difference in the number of seed-predators per capitulum between hybrid and parent *Celmisia* taxa. I collected and sowed seeds from three *Celmisia* hybrids and their parent species in order to test whether hybrids were less fertile than their parent species. I found no evidence to suggest that the seeds of hybrids had lower germination success than those of their parents. Overall I found evidence for only weak prezygotic reproductive isolation and no evidence for postzygotic isolation in the four barriers I examined in *Celmisia*.

CHAPTER ONE

Introduction

Celmisia spectabilis (with *C. graminifolia* in the background)

Hybridisation

Hybridisation occurs in a wide variety of organisms (Huxel 1999; Whitney *et al.* 2010), with around 10% of all animal species and around 25% of all plant species known to hybridise with at least one other species (Mallet 2007). Hybridisation occurs by either homoploid hybridisation, which has no change in ploidy, or allopolyploidy which involves chromosome doubling (Hegarty & Hiscock 2005). Allopolyploid hybrids are instantly isolated from their parents, as hybrids possess a different number of chromosomes to their parent species (Rieseberg 2006; Chapman & Burke 2007). Homoploid speciation is unlikely to occur frequently, as homoploid hybrids need to overcome chromosomal and genetic incompatibilities, without the assurance of reproductive isolation that polyploidy brings (Mallet 2007). Furthermore, as hybrids are often (Hendry, Nosil & Rieseberg 2007; Mallet 2007), but not always (Hegarty & Hiscock 2005), intermediates between their parents, hybrids are likely to be successful only if they inhabit a different niche to their parents (Mallet 2007).

Previously, hybrids were rarely considered to be equally fit or fitter than their parents (Arnold *et al*. 1999). More recently, however, hybrids are being viewed as highly variable (Aldridge & Campbell 2007), and can be as fit or much fitter than their parents (Arnold, Ballerini & Brothers 2012). Such hybrids can be an important starting point for adaptive radiation (Arnold *et a*l. 1999). Although the idea was previously dismissed, the same hybrid combination can be produced on multiple occasions in different locations (Soltis & Soltis 1991; Abbott & Lowe 2004; Hegarty & Hiscock 2005).

Hybridisation is especially widespread in plants (Field *et al.* 2011; Marques *et al.* 2012), and has played an important part in the evolution of most plant species (Carney, Cruzan & Arnold 1994). Some plant families have much higher rates of hybridisation than others (Ellstrand, Whitkus & Rieseberg 1996), with hybrids being particularly common in plant groups endemic to islands (Milne *et al*. 1999). Although plant hybrids are common they are not universal, with hybrids generally being found in a relatively small set of genera (Ellstrand, Whitkus & Rieseberg 1996; Whitney *et al.* 2010). Whitney *et al*. (2010) suggest that the propensity for plants to hybridise may be more to do with plant phylogenetics than with environment factors.

It is becoming apparent that human disturbances are increasing the rates of hybridisation in plants (Rhymer & Simberloff 1996; Milne & Abbott 2008). One disturbance is the breaking down of geographic isolation between species as humans move organisms around the globe or disturb vegetation allowing plants to spread into sympatry (Huxel 1999; Prentis *et al*. 2007). Hybridisation between native and exotic species can result in the evolution of new hybrid lineages (Ainouche *et al*. 2009), increased invasiveness (Ellstrand

2009), or the decline and extinction of a parent species (Levin, Francisco-Ortega & Jansen 1996; Prentis *et al.* 2007). Extinction via hybridisation can occur within five generations, making it an important evolutionary process (Rieseberg 2006; Marques *et al*. 2007).

Speciation

Speciation is the process responsible for biological diversity (Kay 2006), and it is increasingly apparent that hybridisation has played an important role in the production of new species (Nolte & Tauz 2009; Paun *et al*. 2009). Speciation through hybridisation is thought to occur either genetically, through the hybrid acquiring chromosomal differences that prevent reproduction with the parent species (Charlesworth 1995; Rieseberg 2006), or ecologically, through the hybrid accessing different habitats to the parent species (Donovan *et al*. 2010). In the former, allopolyploid hybridisation leads to an increase in species numbers over time (Whitney *et al*. 2010). Polyploidy (without hybridisation) has certainly been important in the evolution of plant species with 40-70% of plant species having a polyploid origin (Mallet 2007). In contrast, ecological hybridisation can lead to adaptive evolution and speciation (Hegarty & Hiscock 2005); therefore, hybridisation is increasingly being considered as a trigger for adaptive radiation events (Mallet 2007; Paun *et al*. 2009; Arnold, Ballerini & Brothers 2012). Adaptive radiations are defined as an increase in diversity within a lineage, usually as a result of divergent selection, leaving a range of closely related species (Givnish 2010). It is thought that closely related species (i.e. those that evolved recently) are more likely to hybridise (Mallet 2007; Paun *et al*. 2009).

Mayr's Biological Species Concept (BSC) is the most widely accepted species concept (Rieseberg & Carney 1998; for a list of alternative species concepts see Coyne & Orr 2004), and it defines species to be interbreeding groups that are reproductively isolated from other such groups (Coyne 1992). However, Mayr applied the BSC in its strictest sense as he believed there was rarely any gene flow between species; therefore, hybrids were rarely formed, had low fitness when they did, and new species rarely arose from hybridisation (Abbott, Ritchie & Hollingsworth 2008). Today, we know that these provisions frequently do not hold true (Rieseberg 1997). However, the BSC has an advantage over other species concepts because it can be tested, and understanding reproductive isolation leads to an understanding of the origin of species (Coyne 1992; Rieseberg 1997). Thus, in order to understand speciation, we must understand how reproductive isolation operates in nature (Sobel & Randle 2009).

Reproductive isolating barriers

Reproductive isolating barriers are any factor that could prevent gene flow between species (Esfeld *et al*. 2009), and are considered crucial for the maintenance of species diversity (Kay 2006; Malone & Fontenot 2008; Marques *et al*. 2012). Isolating barriers are distinct from reinforcement, in which natural selection strengthens prezygotic barriers in response to selection against unfit hybrids (Mallet 2007; Schluter 2009). Reproductive isolation is thought to arise as a by-product of genotypic and phenotypic divergence when species are in allopatry; however, most studies of reproductive isolation are conducted when species have come back into sympatry (Kay 2006). Isolating barriers are classified into those operating before fertilisation (prezygotic) and those operating after fertilisation (postzygotic) (Coyne & Orr 2004). Within each of these categories are a series of factors that could contribute to reproductive isolation (Levin 1971; Coyne & Orr 2004; Nosil, Vines & Funk 2005). Reproductive isolating barriers act in sequence, so that the earlier acting barriers are thought to have a greater influence on whether species are isolated from each other (Coyne & Orr 2004; Martin & Willis 2007; Lowry *et al.* 2008; Dell'Olivio *et* al. 2011).

Prezygotic barriers

Differences in habitat can be an important prezygotic isolating factor in limiting gene flow between species (Levin 1978; Feder, Egan & Forbes 2012), although this is rarely studied (Dell'Olivio *et al*. 2011). In cases where it has been studied, habitat isolation is generally a strong barrier to hybridisation (Nosil, Vines & Funk 2005). Habitat differences do not necessarily need to be on large geographic scales as plants readily adapt to small differences in adjacent habitats (Hendry, Nosil & Rieseberg 2007). Rathcke and Lacey (1985) considered differences in habitat to be more important than differences in flowering phenology, and Dell'Olivio *et al*. (2011) found spatial isolation to be the most important barrier to hybridisation in *Petunia* species. In contrast, parent species in different habitats can still produce hybrids (Cruzan & Arnold 1994); therefore, it is important to study spatial isolation alongside other potential barriers to hybridisation.

If species are reproductive at different times then they are temporally isolated from one another (Lamont *et al*. 2003; Coyne & Orr 2004). In plants, this can be on relatively short timescales (Coyne & Orr 2004). Temporal isolation has not been as thoroughly studied as other barriers, partly because altering an organism's breeding period can be tricky (Coyne & Orr 2004). Differences in flowering phenology are often attributed to underlying habitat differences between plant species, which are known to alter flowering times (Lowry *et al.* 2008). While some studies have found temporal isolation to be unimportant in plants (Rathcke & Lacey 1985), others have found temporal isolation is the most important barrier to hybridisation (Martin & Willis 2007).

Coyne & Orr (2004) define pollinator isolation as different plant species using different pollinators through pollinator specialisation, different visitation frequencies; or different body parts carrying pollen on a single pollinator species. Pollinator isolation can be either ethological (pollinator behaviour) or mechanical (differences in floral and pollinator morphology) (Coyne & Orr 2004). Ethological isolation is often assumed from floral syndromes (Coyne & Orr 2004), although floral syndromes have been shown to sometimes be an inaccurate measure of pollinator type (Robertson, Ladley & Kelly 2005; Ollerton *et al*. 2009). Extreme specialisation is found in some plant-pollinator systems; for example 60-70% of orchid species have a single pollinator species (Cozzolino & Widmer 2005). In most plants, it is the preference and constancy of pollinators that forms the basis of pollinator-mediated reproductive isolation (Kay & Sargent 2009).

Factors such as pollen germination rates, pollen competition and pollen incompatibilities can all contribute to prezygotic reproductive isolation in plant species (Carney, Cruzan & Arnold 1994; Rieseberg, Desrochers & Youn 1995; Campbell *et al*. 2002; Lee *et al*. 2008).

Postzygotic barriers

Postzygotic barriers may have been very important during speciation (Coyne & Orr 2004). These isolating barriers can be intrinsic (independent of the environment), or extrinsic (selection against hybrids as a result of the environment) (Coyne & Orr 2004; Kimball 2008). By their very nature, intrinsic postzygotic isolating barriers assume similar selection against hybrids in all environments (Hendry, Nosil & Rieseberg 2007).

Intrinsic postzygotic isolation takes two forms; hybrid inviability (developmental failure causes partial or full inviability) and hybrid sterility (failure to develop a functioning reproductive system causes partial or full sterility) (Coyne & Orr 2004; Kay 2006). Hybrid sterility is a common form of postzygotic reproductive isolation in both plants and animals (Bomblies 2010).

Extrinsic reproductive isolation includes both failure to adapt to environmental conditions and failure to successfully find a mate (Kay 2006). Hybrids are expected to be less fit (i.e. poorly adapted and selected against) than their parents in either parental environment, as most hybrids are intermediate between their parents (Hendry, Nosil & Rieseberg 2007; Mallet 2007).

Studying reproductive isolation

In order to understand speciation, the role of reproductive isolating barriers in natural systems must be understood first (Rieseberg 1997; Coyne & Orr 2004; Milne & Abbott 2008). While genetic studies can determine how much gene flow occurs between species

(Arnold 1993), or the time since those species diverged (Lockhart *et al*. 2001), it is the reproductive isolating barriers that explain how species can maintain species integrity in sympatry (Johnson *et al*. 1998). Schmeske (2000) lamented the lack of studies investigating ecological components of plant speciation. As most species are reproductively isolated by a suite of barriers (Yang, Gituru & Guo 2007; Marques *et al*. 2012), it is important to study multiple isolating barriers (both pre- and postzygotic) in a single system (Kay 2006; Lowry *et al.* 2008; Widmer, Lexer & Cozzolino 2009). Most studies of reproductive isolating barriers are qualitative (Marques *et al.* 2012), and therefore, cannot be used to assess the relative strength or importance of each barrier or an individual barrier's contribution to total isolation (Lowry *et al.* 2008).

Studies of current reproductive isolating barriers do not provide an indication of how barriers contributed to the original speciation event (Ramsey, Bradshaw & Schemske 2003; Nosil, Vines & Funk 2005; Lowry *et al.* 2008); instead they only explain what currently keeps species separate.

Hybridisation in New Zealand

New Zealand plants are often said to hybridise more readily than plants in other parts of the world (Cockayne 1923; Anderson & Stebbins 1954, Dansereau 1964), although this has more recently been questioned (see below). Heine (1938) attributed high rates of hybridisation to the simple shapes of New Zealand flowers which fail to restrict pollinators. Cockayne (1923) thought hybridisation in New Zealand plants had resulted from human disturbances to natural ecosystems bringing previously isolated species into contact. More likely is that hybridisation in New Zealand plants is a result of many plant species belonging to rapidly radiating groups (Abbott, Ritchie & Hollingsworth 2008), which typically have high levels of hybridisation (Mallet 2007). New Zealand's radiating plant groups all evolved recently with the uplift of the Southern Alps during the Pliocene (5 – 2 mya) (Lockhart *et al.* 2001; Winkworth *et al.* 2002; Linder, 2008). Hybridisation may also be common because climate change events result in habitat transitions (Winkworth *et al.* 2005). Webb and Druce (1984) thought it unlikely that either rapid evolution or disturbances alone could account for the patterns of hybridisation in New Zealand, but noted that both processes together could.

In contrast, Webb & Druce (1984) and Morgan-Richards *et al.* (2009) suggested that New Zealand plants do not hybridise any more frequently than other floras, although no major comparison had been undertaken to validate this. Recently, Wilson & Lee (2012) began such a comparison and concluded that though there are some groups with unusually high levels of hybridisation, overall hybridisation in the New Zealand flora is not particularly high. One group with unusually high rates of hybridisation in comparison to other floras is the

New Zealand Apiaceae (Wilson & Lee 2012), with many combinations of interspecific and intergeneric hybrids in *Aciphylla* and *Anistome* (Webb & Druce 1984). Intergeneric hybrids are frequent in New Zealand Asteraceae (Clarkson 1988; Mckenzie *et al*. 2004), but frequent hybridisation in the Asteraceae is not unexpected as the family is well known for hybridisation worldwide (Ellstrand, Whitkus & Rieseberg 1996). In New Zealand, intergeneric hybrids within the Asteraceae have been attributed to poorly resolved, recent species radiations (Morgan-Richards *et al.* 2009). For example, the Subantarctic daisies form both interspecific hybrids (*Pleurophyllum* species; Figure 1.1), and intergeneric hybrids (e.g. *Damnamenia* and *Pleurophyllum;* Figure 1.1) (Wagstaff & Breitwieser 2004). The genus *Celmisia* is closely related to both *Damnamenia* and *Pleurophyllum* and is well known for its ability to hybridise (Given & Gray 1986).

Figure 1.1: *Damnamenia vernicosa* (left) and *Pleurophyllum criniferum* (centre) inflorescences; intergeneric hybrid occasionally form between these genera, and a *Pleurophyllum criniferum* X *P. speciosum* hybrid (right), Campbell Island.

Celmisia

The genus *Celmisia* Cass. (Asteraceae) is New Zealand's third largest genus (Fenner, Lee & Pinn 2001; McGlone, Duncan & Heenan 2001), with about 60 endemic species in New Zealand and five in Australia (Allan 1961; Given 1969; Fenner, Lee & Pinn 2001). *Celmisia* evolved relatively recently through an adaptive radiation event in the Pliocene (Wardle 1978a), and the species are therefore closely related (Fenner, Lee & Pinn 2001). *Celmisia* are a distinctive part of New Zealand's alpine flora, but several species are also found in coastal locations (Fenner, Lee & Pinn 2001).

Despite being closely related, *Celmisia* exhibit a wide range of leaf morphologies (Allan 1961; Mark and Adams 1995; Appendix 1). All *Celmisia* have radiate capitula with yellow disc florets and white ray florets (Allan 1961; Mark and Adams 1995; Appendix 1). Each capitulum is usually borne singly on its scape, but occasionally a scape will bear several capitula (Allan 1961; Elder 1974).

Celmisia have some economic value as ornamental plants (Fenner, Lee & Pinn 2001; *Joe Cartman, Christchurch City Council, pers. comm.*), and as food for native animals. *Celmisia petriei* is an important component of takahe (*Porphyrio hochstetteri*) diet (Mills *et al.* 1991), and other *Celmisia* are also eaten by kakapo (*Strigops habroptilus*) (Best 1984). Historically *Celmisia* were probably eaten by moa and it has been suggested that *C. lyallii* and *C. petriei* resemble *Aciphylla* species in order to avoid moa browsing (Atkinson & Greenwood, 1989). Today some *Celmisia* species are heavily browsed by invasive ungulates (Christie 1964; Nugent & Challis 1998; Parkes & Forsyth 2008), and both the leaves and inflorescences provide an important food source for native insects (Dugdale 1974).

The genus *Celmisia* is notable for containing species with extreme variability in their inter-annual flowering intensities (Mark 1970; Campbell 1981; Spence 1989). *Celmisia* species are insect pollinated (Fenner, Lee & Pinn 2001), with flies and short-tongued bees being the most frequent flower visitors (Chapter 3). Evidence for self-pollination in *Celmisia* is mixed. Raven (1973), Metcalf (1993) and Given (1968) all describe *Celmisia* as capable of selfing. Metcalf (1993) lists *C. hookeri*, *C. semicordata*, *C. traversii*, *C. gracilenta*, *C. lindsayi*, *C. angustifolia*, and *C. prorepens* as *Celmisia* species known to self-pollinate. In contrast, Thompson (1881) writes that *Celmisia* avoid selfing through protandry (male floral parts are fertile before female). Dichogamy (temporal separation of sexes) is common in Asteraceae, and is an important and often neglected means of preventing self-pollination (Lloyd & Webb 1986). Tests for self-pollination have only been conducted in a small set of New Zealand plants (Webb & Kelly 1993), but as Asteraceae are known for their high rates of self incompatibility, it is likely that many New Zealand species will also display this trait (Newstrom & Robertson 2005).

Various attempts have been made to subdivide *Celmisia* taxonomically. Hooker (in Given 1969) named 25 species in three sub-genera ,and Allan (1961) further divided the species into three sections with nine subsections. Both Hooker and Allan based their sections on morphological characteristics. Given (1969) used 65 morphological characteristics to classify *Celmisia* into six subgenera, one of which was later removed when its sole species (*Celmisia vernicosa*) was reclassed a genus (*Damnamenia*) in its own right (Given 1973) (Figure 1.1). Although some work has been done on creating a molecular

phylogeny for *Celmisia* (*Steve Wagstaff, Landcare Research, pers. comm.*), no published phylogeny exists for *Celmisia* based on anything other than morphological characteristics.

Why study hybridisation in *Celmisia*?

Hybrids within *Celmisia* are well documented, with hybrids being found between many different species (Cockayne 1923; Allan 1961; Given 1969; Wilson 1976; Given & Gray 1986). Interestingly, intergeneric hybrids between *Celmisia* and *Olearia* have also been reported (Burrows 1986; Clarkson 1988). Furthermore, *Celmisia* under cultivation readily hybridise with and without human interference, suggesting that no, or only weak post-pollination, but pre-fertilisation reproductive isolating barriers exist (*Joe Cartman, Christchurch City Council, pers. comm.; Jill Broome, Percy Scenic Reserve, pers comm.).*

There is a discrepancy between the apparent ease with which *Celmisia* form hybrids through hand crosses (*Joe Cartman, Christchurch City Council, pers. comm.*), and the rarity of hybrids in the wild (Mark & Adams 1995), suggesting that some factors are operating to prevent hybridisation or hybrid survival in the wild. Furthermore, *Celmisia* species are noted for their high level of sympatry (Given & Gray 1986); therefore, how can so many closely related species co-exist without losing species integrity? Given (1968) suggested that *Celmisia* are unable to form hybrids because, although sympatry is high for the genus overall, closely related species (based on morphological characteristics) within the genus are rarely found together. Without a better measure of relatedness between *Celmisia* species, Given's hypothesis is not currently testable; however, Given (1968) also suggested a role of phenological separation (staggered flowering) in preventing hybridisation in *Celmisia*.

Study Site

All the field work for my thesis was conducted at Craigieburn Valley Ski Area (42°06'54.55"S, 171°42'18.02"E; Figure 1.2) in the Craigieburn Range, Canterbury, New Zealand.

The Craigieburn Range consists of greywacke and argillite rocks that have been shattered by faulting, glaciations, rivers, and postglacial erosion to form the present day mountain range (Shanks *et al.* 1990; Wardle 1991; Winkworth *et al.* 2005). The highest point on the range is 2195 m and the area receives about 1500 – 2400 mm of rain per year (Shanks *et al.* 1990). While there is no permanent snow in the basins, a snowpack forms in May and thaws from late September, but lingers in some places until mid-January (Shanks *et al*. 1990).

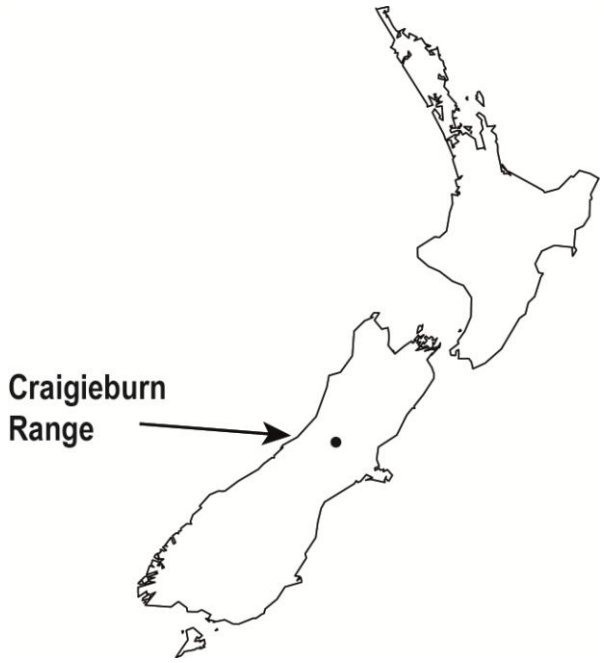

Figure 1.2: All field work for this study was conducted within the Craigieburn Range, South Island New Zealand.

Craigieburn Valley Ski Area consists of two large basins. The ski area infrastructure is based in the South Basin, but skiing also occurs in the unmodified Middle Basin (when snow levels permit). The South Basin has been highly modified by roads, buildings and ski machinery during the formation of the ski area, but areas of unmodified vegetation remain. Regenerating vegetation occurs alongside the day lodge access road. Both basins have depressed treelines (~1200 m a.s.l) as a result of recurring avalanches (*Don Bogie, Department of Conservation, pers. comm.*); this allows a community of alpine plants to extend to lower elevations than normal (Wardle 1991). Shanks *et al*. (1990) give a thorough description of the vegetation types found on the Craigieburn Ranges.

Twelve *Celmisia* species and several hybrids are found in apparent sympatry in the Craigieburn Valley Ski Area. The species are: *C. angustifolia, C. discolor, C. glandulosa, C. graminifolia*, *C. haastii, C. laricifolia, C. lyallii, C. sessiliflora, C. spectabilis, C. verbascifolia, C. viscosa,* and *C. walkeri* (Appendix 1).

Research aims and outline

My thesis aims to quantitatively assess the role of ecological factors that might act as prezygotic and postzygotic reproductive isolating barriers in *Celmisia.*

- In Chapter Two, I examine the role of flowering phenology in *Celmisia* as a potential prezygotic reproductive isolating barrier. I use a null model approach to test for staggered flowering phenologies in *Celmisia*: which is an approach that has not been applied to phenological studies of hybridising taxa before now.
- In Chapter Three, I examine whether the composition and behaviour of insect flower visitors can act as a prezygotic reproductive isolating barrier. I provide the first comprehensive description of flower visitors to *Celmisia.* I also use an information theoretic approach to test whether flower visitors respond to individual floral traits.
- In Chapter Four, I examine two potential postzygotic reproductive isolation barriers (seed predation and germination) in naturally occurring *Celmisia* hybrids. I assess seed predation through the collection and rearing of pre-dispersal seed predators, and germination success with a large greenhouse experiment.
- In Chapter five, I provide a synthesis of the three previous chapters and suggest directions for future work.

CHAPTER TWO

Is flowering phenology a reproductive isolating barrier in *Celmisia*?

Celmisia sessiliflora

Introduction

In plants, the timing of reproductive activity is potentially the most effective reproductive isolating barrier (Martin, Bouck & Arnold 2007), because species that never co-flower cannot form hybrids (Lamont *et al*. 2003). Moreover, early acting barriers have a disproportionately greater influence than later acting barriers due to the linear temporal order in which they act (Coyne & Orr 2004; Lowry *et al*. 2008); temporal differences are especially important for preventing hybrid formation in obligate outcrossers (Armbruster 1986). However, there are fewer studies showing temporal isolation in plants than in animals, and while some studies have shown several examples of temporal differences in plants these differences may have been confounded by those plants also having habitat differences (Coyne & Orr 2004), which often lead to changes in phenology amongst sister species (Lowry, Rockwood & Wiliis 2008). Thus, if no geographic barriers or differences in habitat exist, temporal isolation may be a strong force in preventing hybridisation (Marques *et al*. 2007). Evidence for temporal isolation has been found across a wide variety of plant groups (Lowry *et al*. 2008).

Despite the theoretical importance of phenology as a reproductive isolating barrier, there are a suite of studies that have found little or no temporal differences in flowering times between sympatric species (Vanden Broeck *et al*. 2003; Kay 2006; Marques *et al*. 2007; Lo 2010), leading Widmer, Lexer & Cozzolino (2009) to caution against the emphasis placed on pre-zygotic barriers being the most important for preventing hybridisation.

One of the best tests of whether temporal isolation is important is to remove the isolation and see if hybridisation occurs (Coyne & Orr 2004). For example, anthropogenic disturbance caused two formerly isolated species of Australian *Banksia* to flower synchronously, leading to the formation of hybrid offspring, therefore indicating that temporal isolation had previously been important in preventing hybridisation (Lamont *et al*. 2003). However, tests for differences in the timing of flowering are usually limited to calculating the pair-wise overlap between co-flowering species (e.g. Husband & Sabara 2004; Marques *et al*. 2007). This overlap is then used to calculate a measure of reproductive isolation between the two species (Ramsey, Bradshaw & Schemske 2003; Coyne & Orr 2004). However, few studies test whether these overlaps are different from random at a community scale. I used a null model approach (Pleasants 1980; 1990) that is usually employed to test whether a flowering community has become temporally segregated to avoid competition for generalist pollinators (Boulter, Kitching & Howlett 2006). Use of null models to test for differences in flowering phenology among plant species has been reviewed previously (Gotelli & Graves 1996; Aizen & Vazquez 2006), and evidence for temporal segregation in a population of flowering plants is relatively rare (Aizen & Vazquez 2006; Boulter, Kitching & Howlett 2006). While avoidance of hybridisation is often mentioned as another factor that could contribute to

this pattern (Fleming & Partridge 1984; Fleming 1985; Wheelwright 1985), it is rarely explicitly tested (Borchsenius 2002), and it is seldom tested in a large group of closely related sympatric species.

Studies that have looked at temporal differences in flowering among communities of related species have not set out to explicitly test whether these differences could be preventing hybridisation (Wheelwright 1985; Armbruster 1986; Wright & Calderon 1995; Stone, Willmer & Rowe 1998; Torres & Galetto 2011). Collins & Rebelo (1987) state that hybridisation in sympatric Australian Proteaceae is prevented by staggered flowering phenologies, but do not test this statistically. A series of studies on South American bromeliads (including one study with 42 species) tested the importance of phenology in hybrid prevention (Wendt *et al*. 2001; Wendt *et al*. 2002; Wendt *et al*. 2008), but these studies did not use an appropriate statistical approach (such as those proposed by Pleasants 1990).

Theoretically, alpine plants should flower midway through the summer in order to avoid the disadvantages associated with flowering at either end of the season. Cool temperatures early in the season restrict pollinator availability, leading to pollen limitation, whereas late flowering species rapidly run out of time for seed development, resulting in limited seed set (Molau 1993). It is known that *Celmisia* start producing floral buds in the autumn before flowering, allowing them to elongate and flower earlier in the season (Mark 1970). Therefore, I expected that *Celmisia* would flower synchronously due to the limited flowering time available in a temperate alpine environment, and thus phenology would not be a barrier to hybridisation. Furthermore, individual flowers on alpine plants last longer than those on lowland plants (Primack 1985: Fabbro & Korner 2004), thus increasing the chance of different species overlapping in their flowering times. In contrast, *Celmisia* species are also thought to share the same insect pollinators as each other (Fenner, Lee & Pinn 2001), so it is possible that competition for pollination could lead to a segregated flowering distribution. This chapter aims to test whether differences in flowering times could explain the apparent lack of hybridisation in *Celmisia*, by:

1. Using a null model approach to determine whether *Celmisia* species have a displaced, random or aggregated flowering patterns at three sites within the Middle Basin at Craigieburn Valley Ski Area.
2. Testing whether the previous literature on *Celmisia* hybridisation could be used to predict observed flowering in Craigieburn *Celmisia*.
3. Testing whether flowering phenologies in *Celmisia* can be explained by their relatedness.

Methods

Field methods

To record the flowering of the 12 sympatric species of *Celmisia* found in the Middle basin, three 30 m permanent transects were established along horizontal contours, with one at each of the following altitudes; 1250, 1320, and 1390 m a.s.l (transects are hereafter referred to by altitude) (Figure 2.1). In order to sample the same patch of *Celmisia* on each repetitive visit the transects were marked with permanent stakes and a tape was run along these on every visit. These transects were surveyed approximately weekly for the duration of the flowering season and the number of open capitula from every *Celmisia* species within one metre either side of the transect was recorded.

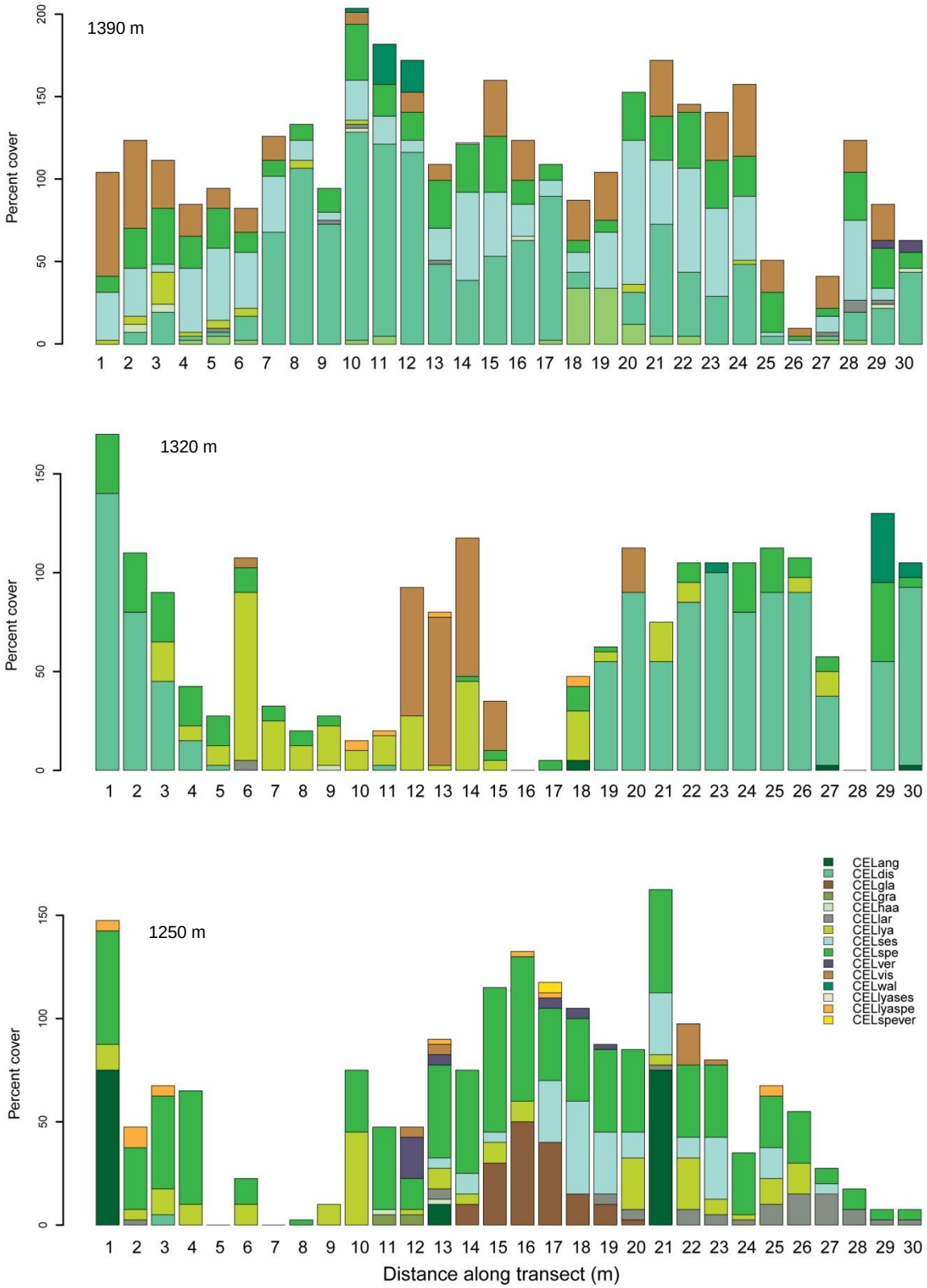

Figure 2.1: Percent cover of each *Celmisia* taxon (in 2 m^2) every metre along the 30 m transect at each altitude. CELang = *C. angustifolia*, CELdis = *C. discolor*, CELgla = *C. glandulosa*, CELgra = *C. graminifolia*, CELhaa = *C. haastii*, CELlar = *C. larcifiolia*, CELlya = *C. lyallii*, CELses = *C. sessiliflora*, CELspe = *C. spectabilis*, CELver = *C. verbascifolia*, CELvis = *C. viscosa*, CELwal = *C. walkeri*, CELlyases = *C.* lyallii X *C.* sessiliflora, CELlyaspe = *C. lyallii* X *C. spectabilis*, CELspever = *C. spectabilis* X *C. verbascifolia*.

Data analysis

The number of capitula open for species *x* at each visit was transformed into a proportion of all capitula produced by species *x* on that transect over the whole season. I calculated the overlap for each *Celmisia* species with every other species along each transect using the using the Schoener formula as given by Pleasants (1980).

$$1 - \frac{1}{2} \sum_{k=1}^{n} |pik - pjk|$$

This gives a measure of niche overlap between two species where p_{ik} and p_{ij} are the proportion of flowers of species *i* and *j* open on the *kth* day, and *n* is the total number of days in the flowering season. A value of 0 indicates no overlap, whereas a value of 1 indicates complete overlap. I calculated the mean pair-wise flowering overlap for each transect by averaging the mean overlap of all possible species pairs that produced flowerheads. Overlaps greater than 0.5 indicate a weak reproductive isolating barrier, while those greater than 0.75 indicate a very weak barrier (Lowry *et al*. 2008).

To test whether flowering distributions in *Celmisia* were segregated (low flowering overlaps between species; unlikely to form hybrids), synchronous (large flowering overlaps between species; hybridisation is possible), or random, I employed the null model approach of Pleasants (1980; 1990). Use of null models in examining phenology distributions has been reviewed previously (Pleasants 1990; Gotelli & Graves 1996; Aizen & Rovere 2010). My null model kept the shape of each observed flowering distribution but moved each species flowering curve randomly and independently of each other within a three month summer season. I avoided the problems of an artificially shortened flowering season (see; Ashton, Givnish & Appanah 1988; Gotellii & Graves 1996), by allowing the null model's flowering season to extend a few weeks longer than the observed flowering season at both ends of the season. This also served to crudely allow for the seasonality experienced by alpine plants in that the flowering period in the null model was approximately the length of the natural summer season (Aizen & Vazquez, 2006). I let the null model run for 1000 iterations, and compared the mean pair-wise overlap value of each transect to the means generated from each iteration of their respective null model. An observed mean overlap smaller than the median 97.5th percentile (i.e. less than 97.5% of the null model means) indicated that the flowering distribution was segregated, and conversely a mean larger than the median 2.5th percentile (i.e. bigger than 97.5% of the null model means) indicated that species were flowering synchronously (Boulter, Kitching & Howlett 2006).

From the literature I created an index of hybrid frequency for all possible combinations of species pairs from the *Celmisia* at the study site. I ranked these pairs as 0 (no records of hybridisation); 1 (very few records of hybridisation); or 2 (frequent records of hybridisation) to

create the index. Using this index I performed a one way ANOVA using the index as a predictor for the observed flowering overlap values at Craigieburn, to see if species which often hybridise have more overlap in flowering times on average.

Using Given's (1969) classification of subgenera in *Celmisia* I was able to test whether closely related species (i.e. those sharing the same subgenus) were likely to have greater phenology overlap values than those that were not closely related (i.e. in different subgenera). I performed a one way ANOVA using same or different subgenus as the predictor for observed phenology overlap. All statistical tests were performed in the programme 'R' version 2.14.2 (R Development Core Team 2012).

Results

In the 2010-2011 season, *Celmisia* at Craigieburn flowered from November 2010 until March 2011. All 12 *Celmisia* species flowered on at least one of the transects (Figure 2.2). Most species reached their flowering peak in January (Figure 2.2).

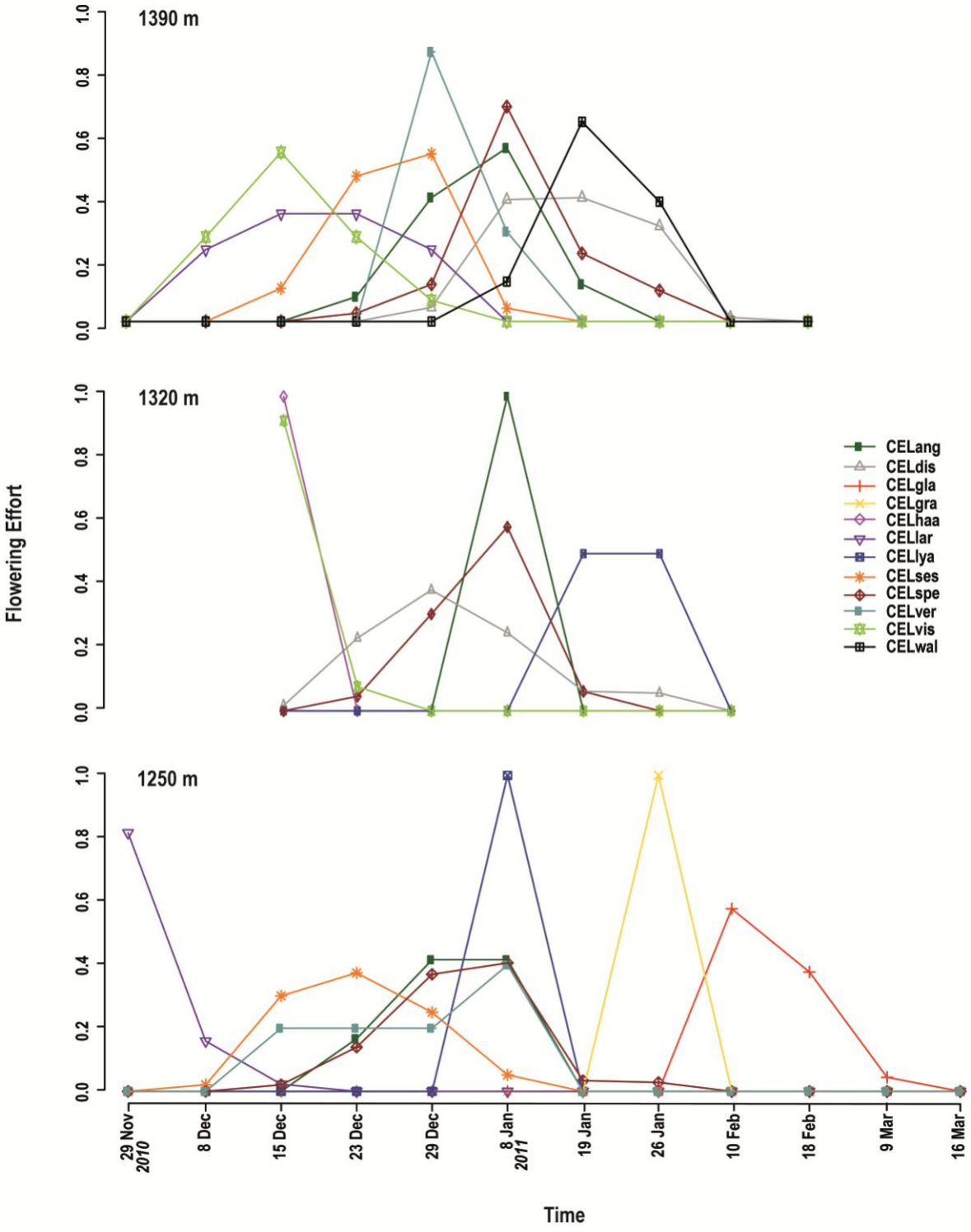

Figure 2.2: Flowering phenology for 12 *Celmisia* species at three different altitudes (a: 1390, b: 1320, c: 1250 m a.s.l.) at Craigieburn Middle Basin. Flowering effort was measured as the capitula seen for each species on any given sample day as a proportion of the total number of capitula produced throughout the entire season. Each of the 12 *Celmisia* species is represented here by a different symbol and coloured line.

There was very little difference in flowering between altitudes for species found along multiple transects, with flowering being delayed at the most by 4.7 days at higher altitudes.

Overlap values varied from 0 (no overlap), to 0.923 (of a possible 1) for *C. viscosa* – *C. haastii* (Table 2.1). *Celmisia glandulosa* was the only species to have zero overlap with all other species (although *C. graminifolia* barely overlapped with any other species) and most pair-wise combinations had low overlap values (Table 2.1). Overall, 20% of the overlap values could not be calculated (NA); 29% were 0; 41% were between 0<0.05; 7% were between 0.5<0.75; and 3% were ≥0.75

Table 2.1: Pair-wise flowering overlaps for all *Celmisia* species seen flowering at Craigieburn Valley Ski Area over the 2010/2011 flowering season, where NA means species were not found together on the same transect. For species pairs that occurred on multiple transects, the mean overlap is shown here.

	CELang	CELdis	CELgla	CELgra	CELhaa	CELlar	CELlya	CELses	CELspe	CELver	CELvis	CELwal
CELang	1.000											
CELdis	0.365	1.000										
CELgla	0.000	NA	1.000									
CELgra	0.000	NA	0.000	1.000								
CELhaa	0.000	0.017	NA	NA	1.000							
CELlar	0.134	0.040	0.000	0.000	NA	1.000						
CELlya	0.208	0.119	0.000	0.000	0.000	0.000	1.000					
CELses	0.460	0.077	0.000	0.000	NA	0.318	0.052	1.000				
CELspe	0.738	0.439	0.000	0.028	0.000	0.342	0.234	0.313	1.000			
CELver	0.681	0.290	0.000	0.000	NA	0.111	0.400	0.578	0.557	1.000		
CELvis	0.064	0.067	NA	NA	0.923	0.794	0.000	0.386	0.064	0.059	1.000	
CELwal	0.215	0.721	NA	NA	NA	0.000	NA	0.037	0.387	0.111	0.000	1.000

Where: CELang = C. angustifolia; CELdis = C. discolor; CELgla = C. glandulosa; CELgra = C. graminifolia; CELhaa = C. haastii; CELlar = C. laricifolia; CELlya = C. lyallii; CELses = C. sessiliflora; CELspe = C. spectabilis; CELver = C. verbascifolia; CELvis = C. viscosa; CELwal = C. walkeri.

The species with the most overlaps were *C. spectabilis* and *C. discolor* (overlapped with nine other species), closely followed by *C. sessiliflora* and *C. angustifolia* (overlapped with eight other species) (Table 2.1). The mean phenology overlaps for each species across all transects are all very low (Table 2.2).

Table 2.2: Average phenology overlaps for all species across all transects.

Celmisia species	Mean flowering overlap
C. angustifolia	0.26
C. discolor	0.24
C. glandulosa	0
C. graminifolia	0.004
C. haastii	0.19
C. laricifolia	0.17
C. lyallii	0.10
C. sessiliflora	0.22
C. spectabilis	0.28
C. verbascifolia	0.28
C. viscosa	0.29
C. walkeri	0.21

Flowering distributions at the 1250 m a.s.l. and 1390 m a.s.l. were no difference to random, as both these transects had a mean pair-wise overlap that was within 97.5% of the means produced by the null model (Table 2.3). The 1320 m a.s.l. transect however, had an observed overlap that was smaller than 97.5% of the null model means (Table 2.3), which indicates that these *Celmisia* had a segregated flowering pattern in the 2010-2011 season.

Table 2.3: Results of the null model analyses.

Altitude	Number of species	Actual mean overlap	Randomisations		Distribution
			2.5 percentile	97.5 percentile	
1250	8	0.18684	0.048258	0.193284	Random
1320	6	0.016546	0.033614	0.229628	Segregated
1390	8	0.302419	0.115806	0.317772	Random

Forty-six *Celmisia* species pairs had no known hybrids, 12 species pairs had few known hybrids, and seven had frequently reported hybrids. Flowering overlaps were significantly greater in *Celmisia* species pairs with known hybrids than in pairs with few or no known hybrids (Table 2.4).

Table 2.4: ANOVA table for the hybridisation Index test

	df	Sum sq.	Mean sq.	F-value	p-value
Hybridisation Index	2	0.48	0.24	4.31	0.02
Residuals	50	2.79	0.06		

Celmisia species at Craigieburn include members of four Celmisia subgenera. These are; Lignosae (*C. angustifolia*, *C. discolor*, *C. walkeri*, *C. haasti*, and *C. viscosa*), Glandulosae (*C. glandulosa*), Pelliculatae (*C. spectabilis*, *C. lyallii*, and *C. verbascifolia*), and Celmisia (*C. sessiliflora*, *C. laricifolia*, and *C. graminifolia*) (Given, 1969). Observed flowering overlaps were not significantly more different between species pairs that were in the same or different subgenera (Table 1.5). The mean phenology overlap within each subgenera was: 0.106 for Celmisia; 0 for Glandulosae; 0.26 for Lignosae, and 0.40 for Pelliculatae.

Table 1.5: ANOVA table for the subgenera test.

	df	Sum sq.	Mean sq.	F-value	p-value
Subgenera	1	0.09	0.09	1.37	0.25
Residuals	51	3.18	0.06		

Discussion

Despite the relatively short length of the alpine flowering season, the null model test suggests that some temporal segregation exists among species in this *Celmisia* community. Although only one transect was significantly temporally segregated, the other two were not significantly aggregated. Additionally, I found that parental species of well known hybrids had significantly higher observed flowering overlaps than species pairs with few or no known hybrids, therefore suggesting that similar patterns of flowering have occurred elsewhere in the past. Furthermore, observed flowering patterns in *Celmisia* is not a direct result of shared ancestry because I found no significant difference in observed flowering overlap between *Celmisia* species pairs that were in the different subgenera. Together, these tests indicate that differences in flowering times may have a slight role in the reproductive isolation of some *Celmisia* species. A previous study on the phenology of plants in the Craigieburn Ranges did not find any evidence for temporal segregation in flowering times (Spence 1989). However, Spence's work examined a whole assembly of plant species instead of focusing on one genus. Closely related species are likely to share floral characteristics that could lead

to species evolving divergent flowering times in order to avoid competition for generalist pollinators (Boulter, Kitching & Howlett 2006).

Finding temporal segregation among potentially hybridising plants indicates that plants are unlikely to form hybrids in the present, but does not mean that plants have evolved to be temporally segregated to avoid hybridisation in the past. Although there are some cases where plant populations have evolved different flowering times to avoid hybridisation (Mcneilly & Antonovics, 1968; Lamont *et al*. 2003; Ferriol *et al*. 2009) these generally involve a drastic change in the underlying habitats for these plants. Some divergent flowering times have also been shown to have an underlying genetic basis (Martin, Bouck & Arnold 2007). As previously mentioned, the best test of whether divergent flowering times prevent hybridisation is to remove the isolation and see if hybrids readily form (Lamont *et al*. 2003). However, in the absence of such a test there are several other factors that could be driving the flowering patterns seen amongst *Celmisia*.

Competition for pollinators

Communities undergoing strong competition for pollinators might evolve temporally segregated flowering distributions (Aizen & Rovere, 2010). However, very few studies have ever found this (Boulter, Kitching & Howlett 2006; Aizen & Rovere, 2010). Alternatively plants can undergo character displacement to avoid competition with co-flowering species (Aizen & Rovere, 2010). The evolution of character displacement assumes some level of specialisation by the flower visitors which may not be found in the New Zealand alpine flora (Campbell *et al*. 2010; Chapter three). Plants with similar morphologies should experience especially strong pollinator competition (Boulter, Kiching & Howlett 2006; Stevenson *et al*. 2008). Patterns that we observe today may reflect a community that has already undergone competition that removed those that were unable to compete successfully (Pleasants 1980).

Showing that plants are temporally segregated does not prove that competition is responsible for shaping that pattern. For plants, some measure of the plants doing poorly (e.g. pollen limitation) also needs to be shown in the presence of competition. Aizen & Vazquez (2006) found evidence for temporal segregation in a temperate plant community, and a follow up study (Aizen & Rovere 2010) showed that this temporal difference could be explained by avoidance of pollinator competition.

Features that reduce competition for pollinators should also reduce hybridisation (Collins & Rebelo 1987), because if displacement in flowering times occurs then the chance of hybridisation is subsequently lowered. Additionally, Levin (1971) argued that selection against competition for pollination is selection against hybridisation, as selection against competition is selection on a reproductive trait.

Phylogenetics

Related species are likely to have similar flowering patterns (Kochmer & Handel 1986; Smith-Rameirez, Amensto & Figueroa 1998; Aizen & Vazquez 2006; Boulter, Kitching & Howlett 2006). Stevenson *et al*. (2008) suggest that phylogenic constraints (or shared adaptive responses to climatic cues) may be more important than competition for pollinators in shaping plant phenologies. However, studies in which displaced flowering times have been convincingly shown are those that studied a group of closely related plant species (Ashton, Givnish & Appanah 1998; Stone, Willmer & Rowe 1998; Lobo *et al*. 2003). While each of these studies used closely related species, they did not test whether avoidance of hybridisation could also be contributing to the displaced flowering patterns. Lobo *et al*. (2003) and Stone, Willmer & Rowe (1998) do not consider hybridisation, whereas Ashton, Givnish & Appanah (1988) dismiss temporal differences as an isolating barrier because strong post zygotic isolating barriers exist for their study species. Avoidance of pollen discounting (the loss of pollen that could have been available for outbreeding (Lloyd 1992; Barrett 2002)) and stigma clogging (the blocking of the stigma by foreign pollen which prevents subsequent fertilisation (Brown & Mitchell, 2001)) also benefit from temporal segregation of flowering in sympatric species. Pollen discounting and stigma clogging could explain why temporal segregation exists despite the presence of strong post-zygotic isolating barriers.

To test the effect of phylogeny on flowering times requires a well resolved phylogeny. For example, closely related South American Myrtaceae tend to flower under similar climatic conditions (Staggemeier, Diniz-Filho & Morellato 2001). No such phylogeny currently exists for *Celmisia* (Fenner, Lee & Pinn 2001), therefore the effect of phylogeny on *Celmisia* flowering times cannot be more accurately tested.

Abiotic factors

Weather conditions are potentially the most important factors determining flowering times in individual species (Aizen & Vazquez 2006). Consequently, in temperate environments evidence of displaced flowering phenologies is rare because of the disproportionate influence of climatic factors on flowering (Aizen & Vazquez 2006). A large study of Asteraceae found that many had limited flowering distributions because of both climatic factors and phylogenetic histories (Torres & Galetto 2011). Temperate species are expected to be more constrained by temperature changes than tropical species due to the extreme changes in seasonality they are exposed to (Aizen & Vazquez 2006). Temperature, photoperiod, and rainfall are all thought to be very important in determining the flowering phenology of plants (Torres & Galetto 2011).

Flowering in some New Zealand alpine species is restricted by the length of the summer snow pack (Wardle 1978b). The length of the summer snow pack varies annually (Shanks *et al*. 1990), and flowering in such species can occur on the edge of the retreating snow pack or shortly after the disappearance of the entire snow pack (Wardle 1978b). In New Zealand *Celmisia* this is true of *C. hectori* (Campbell 1981), and *C. haastii* (Mark 1970). An Australian species of *Celmisia* (*C. pugioniformis*) is known to flower simultaneously within a population regardless of when individual plants were released from the snowpack, due to changes in photoperiod controlling flowering (Venn & Morgan 2007). Furthermore, many *Celmisia* are highly variable in their inter-annual flowering behaviour (Mark 1970), with some species recorded to have up to 13 years in between peak flowering events (Campbell 1981). Autumn initiation of floral buds is the norm in *Celmisia*, but the majority of floral buds do not develop further the following flowering season (Mark 1970).

Conclusions

I found some evidence of temporal segregation between species of *Celmisia*, but not enough to conclude that it is acting as a strong reproductive isolating barrier. The null model approach was useful to test for patterns in flowering amongst the *Celmisia* community at Craigieburn. The use of null models should be more commonly applied to test for temporal isolation in other groups of potentially hybridising plants as they provide a more rigorous test for phenological overlap than is commonly applied in hybridisation studies. Access to previous records of *Celmisia* hybrids allowed more confidence to be placed in the results of the null model and provided a suggestion that similar flowering patterns had occurred elsewhere in the past.

It is extremely hard to disentangle the effects of seasonality, phylogeny, avoidance of hybridisation, and pollinator competition from each other, as individual plants respond to a variety of cues before flowering (Boulter, Kitching & Howlett 2006). Further work on this system should focus on testing the flowering patterns of *Celmisia* against a better measure of their relatedness. More consideration should also be given to the role of abiotic factors, as it is highly probable that these are the biggest influence on flowering in *Celmisia* given the temperate alpine environment they inhabit. Therefore, while this study found some evidence of slight temporal displacement between some species of *Celmisia* it is certainly not the most important factor in preventing hybridisation. In this case, temporal isolation should be thought of as a "leaky barrier" (Widmer, Lexer & Cozzolino 2009), and is therefore only part of the answer as to why *Celmisia* hybrids are not encountered more often in the wild.

CHAPTER THREE

Do flower visitors prevent hybridisation in *Celmisia*?

C. angustifolia

C. discolor

C. glandulosa

C. graminifolia

C. haastii

C. laricifolia

C.lyallii

C. sessiliflora

C. spectabilis

C. verbascifolia

C. viscosa

C. walkeri

Introduction

An enduring idea in plant speciation is specialisation by pollinators drives the divergence of floral forms and thus causes reproductive isolation (Jones 2001). The role of pollinators as agents of reproductive isolation is the best studied hypothesis of hybridisation barriers in plants (Kay 2006), perhaps because of the long held idea of pollinator specialisation (Lowry *et al*. 2008). More recently, however, pollinator communities are increasingly being regarded as highly generalist (Waser *et al*. 1996; Kephart & Theiss 2004; Lazaro, Lundgren & Totland 2009). There are clear benefits to plants utilising multiple pollinators, primarily greater reproductive assurance through multiple potential pollinators (Kephart & Theiss 2004; Pohl, Van Wyk & Campbell 2011). Pollinators are expected to be generalists when floral rewards are similar across all plant species and when travel between plant species is costly (Waser *et al*.1996). Conversely, the parallel radiation of angiosperms and pollinators, as well as plants requiring biotic pollination being more speciose than those with abiotic pollination mechanisms, suggests that pollinators did contribute to the speciation of a vast number of plant groups (Jones 2001). Furthermore, avoidance of pollinator sharing has benefits other than hybrid prevention. For example, shared pollinators could contribute to pollen discounting (Feinsinger 1987), and increased stigma clogging (Yang, Gituru & Guo 2007; Pohl, Van Wyk & Campbell 2011), both of which lower plant reproductive fitness. What role pollinators continue to play in plant reproductive isolation today is less clear (but see; Godsoe *et al.* 2008).

A recent review of reproductive isolation mechanisms ranked pollinator-mediated reproduction higher than differences in flowering phenology (Lowry *et al*. 2008). Work on *Ipomopsis* (Campbell, Waser & Melandez 1997), *Costus* (Kay 2006), *Petunia* (Dell'Olivio *et al*. 2011), and *Chamerion* (Husband & Schemske 2000) all found pollinator-driven reproductive isolation. However, these studies all had strong colour and/or morphological differences between species, a feature that is often linked with reproductive isolation between plant species (Grant 1994; Charlesworth & Charlesworth 2000). Other studies have found evidence of pollinator-mediated reproductive isolation, but it was not considered strong enough to prevent hybridisation on its own (Goulson & Jerrim 1997; Marques *et al*. 2007; Martin, Bouck & Arnold 2008). Xu *et al*. (2011) suggest that although pollinator-mediated isolation is often strong, few studies have tested that this barrier maintains species integrity on its own.

Coyne & Orr (2004) suggested that pollinator isolation could be inferred from a lack of natural hybrids where co-flowering animal-pollinated plant species are found sympatrically. However, such an inference is unrealistic as many other factors besides pollinator isolation could also be preventing hybridisation. There are several components of pollinator-mediated

reproductive isolation. Firstly, plant species may be completely isolated via extreme specialisation in their pollinators. Extreme specialisation is the exception rather than the rule (Lazaro, Lundgren & Totland 2009), but this form of reproductive isolation has been found in sexually deceptive orchids (Bower 1996; Abbott, Ritchie & Hollingsworth 2008; Xu *et al*. 2011), figs and fig-wasps (Coyne & Orr 2004), and yucca and yucca moths (Godsoe *et al*. 2008). Secondly, plants can share pollinators, but may be mechanically isolated from each other via the placement of pollen on different body parts of the pollinator (Kay 2006). Thirdly, plants may share the same community of pollinators, but the pollinators may display strong preferences (defined as the overexploitation of one species in the presence of other species) for some species over others, therefore reducing the chance of between-species visits (Aldridge & Campbell 2007). Lastly, pollinators may display constancy (defined as the tendency of a pollinator to make consecutive visits to the same species, whilst disregarding other species in the course of its foraging bout) (Aldridge & Campbell 2007).

Alpine pollinators in New Zealand have long been regarded as generalist foragers (Heine 1938; Primack 1978; Godley 1979). Consequently, the preponderance of white-flowering alpine plants was attributed to the lack of long-tongued bees and the generalist behaviour of flower visitors (Heine 1938). Although 60% of the New Zealand flora (Webb & Kelly 1993; Korner 1999) and 78% of the alpine flora (Wardle 1991) have white flowers, most flowers are not entirely white and often feature yellow colourations (Godley 1979; Mark & Adams 1995). Godley (1979) cautioned that the ultimate cause of white coloured flowers should be examined genus by genus, and Newstrom & Robertson (2005) supported this by stressing that much remained unknown. A recent series of papers (Campbell *et al*. 2010; 2012) have shown that New Zealand alpine pollinating insects do display foraging behaviours that depart from the previously assumed generalist behaviour.

Mechanical isolation in *Celmisia* can be dismissed as all *Celmisia* have open access inflorescences (Mark & Adams 1995; Newstrom & Robertson 2005; Appendix 1); therefore, placement of pollen on different parts of insect visitors is unlikely. If flower visitors to *Celmisia* behave as generalists (as most of the literature suggests) then it is unlikely that pollinators could be preventing hybridisation. Conversely, in the context of recent findings (Campbell *et al.* 2010; 2012), insects might show preferences for some *Celmisia* species over others, but whether such preferences translate into constancy is less clear. This chapter asks:

1) Are *Celmisia* species morphologically different to each other (as slight morphological differences might drive insect preferences)?

2) Can floral characteristics predict insect behaviour?

3) Do *Celmisia* share flower visitors?

4) Do flower visitors have a preference for one species over another?

5) Do flower visitors show constancy in their visitation behaviour?

Methods

Experimental set-up

Following the methodology of Campbell *et al*. (2010), pair-wise *Celmisia* arrays were used to observe the behaviour of insect flower visitors. Each array contained eight capitula of two species randomly placed into 16 water filled vials (Figure 3.1).

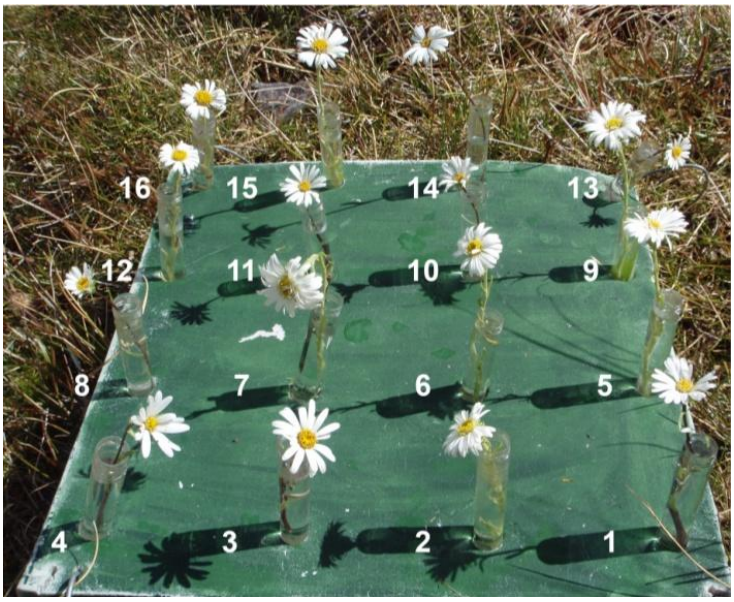

Figure 3.1: A *Celmisia* observation array, with numbers indicating the position of each capitulum inside the array. Species pictured are *C. angustifolia* (dark stems) and *C. discolor* (light stems).

Capitula were carefully picked from the base of the rosette so the scapes retained any natural height variation both within and between species. Arrays were placed in the environment the capitula were sourced from, so that they were near (5 - 10 m) but not in a patch of the same species. This heightened the chance that visiting insects were not naive to the species they were being presented with (Campbell *et al*. 2010; Pohl, Van Wyk & Campbell 2011; Campbell *et al*. 2012). *Celmisia* species pairs were chosen by selecting species that were flowering heavily next to each other in multiple locations across the field site (Table 3.1).

Table 3.1: *Celmisia* species used in the pair-wise arrays with the number of replicates and hours observed.

Array species	Replicates	Hours observed	Mean visits/min
C. angustifolia : *C. discolor*	10	8.22	0.04
C. angustifolia : *C. graminifolia*	10	7.69	0.05
C. angustifolia : *C. viscosa*	7	8.07	0.02
C. graminifolia : *C. spectabilis*	10	6.19	0.08
C. lyallii : *C. spectabilis*	9	9.56	0.03
C. sessiliflora : *C. spectabilis*	10	11.30	0.02

Some species were not represented due to natural rarity in the environment (e.g. *C. verbascifolia*), others due to poor or early flowering (e.g. *C. haastii, C. laricifolia*), some were only represented in a single array because of limitations in how many capitula became available. Each new replicate array was located somewhere different from previous arrays. Arrays were observed for a minimum of 30 minutes and up to a maximum of two hours depending on the rate of insect visitation. Capitula did not visibly wilt over this period. All insects that entered the array and fed from a *Celmisia* capitulum were observed and their visitation pattern recorded until they left the array. It is possible that the same insect was followed through the array on multiple occasions, but because many insects of the same species were often observed visiting the array simultaneously I am confident that the majority of insects recorded visiting the array were different individuals (Campbell *et al*. 2010). Arrays were terminated at 30 minutes if 15 or more insect visitors had been observed, or if no insects had been observed visiting the arrays. Observations continued past 30 minutes if more than one but less than 15 insects had visited. Arrays were then either terminated after 15 insects had visited or at two hours regardless of the number of visitors. If the fifteenth visitor was still on the array at 30 minutes and if other insects arrived, arrays were watched until no insects were on any of the *Celmisia* capitula. Array observations took place between 1200 and 1500 m a.s.l. in both basins of Craigieburn Valley Ski Area.

Insects were given descriptive tag-names in the field and representative samples of all species were collected or photographed for later identification. This collection took place throughout the field season and insects were collected from arrays at the end of observation periods or from other plants as the opportunity arose. Almost all insects were placed in their respective families, while most insects were identified to genus, with a few identified to species (Table A2.1).

Upon the completion of each array, measurements (mm) of floral characteristics were taken from all capitula used in the array to be used as predictors of insect visitation in later

analysis. These were: height of the capitula (from the base of the array to the centre of the capitulum), diameter of the entire capitulum including ray florets (at the widest point), diameter of the disc florets (at the widest point), and the phenological stage of the capitulum (Figure 3.2; 3.3). The ratio of ray to disc florets (white to yellow) was calculated (as per Fenner, Lee & Pinn 2001).

Figure 3.2: Measurements of *Celmisia* floral characteristics: a) scape height (mm); b) capitulum diameter (mm); disc diameter (mm). Species pictured are *C. angustifolia* (a), and *C. spectabilis* (b and c).

Figure 3.3: The four phenological stages in *Celmisia*: a) only ray florets open – "female"; b) ≤ 50% of the disc florets open – "male"; c) >50% of the disc florets open – "male"; d) disc florets displaying stigmas – "female". All capitula pictured are *C. angustifolia*.

The phenology of each capitulum was scored using an index system that I created. *Celmisia,* like other daises, have florets that open sequentially from the outside of the capitulum through to the centre of the disc (Harris 1995). I classed this sequential opening of the capitulum using four levels; 1) only ray florets open, 2) ≤50% of the disc florets open and bearing pollen, 3) >50% of the disc florets open and bearing pollen, and 4) the disc florets display stigmas (Figure 3.3). As the first and last stages of this classification system are female only (Given 1968), the phenology index can also be treated as a male/female measure of the capitulum rather than just a measure of the capitulum's age.

Statistical analysis

All statistical analyses were performed using the programme 'R' version 2.14.2 (R Development Core Team, 2011). Analyses specific to each question are outlined below.

Are* Celmisia *species morphologically different?

One way ANOVA tests were performed to test whether individual floral characteristics differed between *Celmisia* species. I performed a non-metric multidimensional scaling (NMDS) ordination analysis using the R package 'vegan' (Oksanen *et al*. 2012) to test whether the seven species of *Celmisia* were morphologically different across all measured traits. The NMDS approach preserves the rank ordering of the distances in a low dimensional space rather than preserving the original distances as in PCA-type approaches (Zuur, Ieno & Smith 2007). Points closer to each other in the NMDS ordination plot are groups that are more similar than others (Mahecha *et al*. 2007; Zuur, Ieno & Smith 2007).

Do* Celmisia *species share the same flower visitors?

I ran another NMDS ordination to see if the *Celmisia* in my study utilised a different flower visitor community to each other. I used each *Celmisia* species at each array as replicates in the analysis (i.e. there were seven replicates of *C. viscosa* and 25 replicates of *C. angustifolia*). I used the frequency of visits by each insect genus to each species at the array as a measure of the insect community.

Do insects show a preference for* Celmisia *species?

Chi-square tests of independence were used to assess the preference of insects for a *Celmisia* species. All the observed insect taxa were clumped together for the first test of visitor preference and then insects were separated out into the four most abundant families (from all observations). These were one bee family, Colletidae, (genus: *Leioproctus*) and three fly families, Empididae (genus: *Hilaria*), Syrphidae (genera: *Allograpta, Eristalis, Helophilus, Melangyna,* and *Merodon*), and Tachinidae (genera: *Protohystrichia*, and some unidentified). The preference of all insects was tested using the first flower visited as they

entered the array against a null hypothesis of equal visits to both species. Each array type was analysed separately.

To test for preferences during foraging bouts (with at least two capitula visited), I used the *plotmeans* function from the R package 'gplots' (Warnes 2011). I plotted the proportion of visits by each insect family to the preferred species from the chi-square tests (above). By including the 95% confidence interval in these graphs, I could see whether individual insect families had a significant preference to either species during foraging bouts (as per Campbell *et al*. 2010).

Can floral characteristics predict the preference of insect visitors to* Celmisia*?

To test whether insects based their plant species preferences on any of the measured floral characteristics, I used an information theoretic approach to evaluate the strength of multiple predictors of insect behaviour. Information theoretic (IT) approaches provide an alternative to null hypothesis testing, and allow several competing hypotheses to be tested, whilst simultaneously dealing with model fit and uncertainty (Meyer *et al*. 2008; Garamszegi 2011).

I used second-order AIC (AICc) because this is advised for smaller sample sizes and it begins to approximate AIC as sample size increase (Burnham & Anderson 2002). The change in AICc (∆AICc) can then be calculated, whereby the model in the candidate set with the most support is given a ∆AICc of 0. A strength of the IT approach is the ability to calculate model weights (w_i). These give a probability that model *i* is the best model in the candidate set (Anderson 2008).

Inferences can be based from a single top model if the $w_i > 0.90$, or from a set of models. Model weights are additive, and a therefore a confidence set can be established by summing models (Burnham, Anderson & Huyvaert 2011). I used a 95% confidence set from which to base my inferences (Burnham & Anderson 2002). However, as a general rule of thumb, models with a ∆AICc ≤ 2 are models with a high level of support; those with a ∆AICc of 2 ≤ 6-7 have a moderate level of support; those with a ∆AICc > 11 have little support, and those with a ∆AICc >20 essentially have no support (Burnham, Anderson & Huyvaert 2011).

I used generalised linear mixed models (GLMMs) for model selection as these incorporate both random and fixed effects (Zuur *et al*. 2009). GLMMs provide a further advantage in that they do not require the underlying data to be normally distributed (Grueber *et al*. 2011). For all models the random effect was array nested within location, where location was either the Middle or South Basin of Craigieburn Valley Ski Area. These models were run using the 'lme4' package in R (Bates, Maechler & Bolker 2011), and the full set of candidate models can be found in Appendix 3. Model selection was run in the R package 'AICcmodavg' (Mazerolle 2012).

I tested for collinearity in my model parameters using the *pairplot* function in the R package 'AED' (Zuur 2010). Not all array types had enough insect foraging bouts (replicates) to run all of the model combinations, and the *pairplot* function indicated slight collinearity for two of the parameters (height and size) in the *C. sessiliflora*: *C. spectabilis* array; therefore some arrays had a modified set of candidate models (Appendix 3; Figure A3.1).

To assess model fit I included an intercept-only model in my candidate model set. Comparing where this model is ranked in comparison to the other models provides an indication of whether the predictors are providing useful information. If the intercept-only model was in the 95% confidence model set then there is evidence that the models are not representing the data well. An adjusted R^2 is often calculated to give a measure of model fit, but I could not use this method as it is not resolved for the inclusion of a random effect (Grueber *et al*. 2011). The Akaike weights generated in the model selection process provided a further measure of model uncertainty (Meyer *et al*. 2008). When the top model has a low weight or there are several models in the 95% confidence set, there is evidence for model uncertainty (Anderson 2008).

In cases where a high degree of model uncertainty was evident, I employed model averaging (Burnham & Anderson 2002) using the R 'MuMIn' package (Barton 2012). Model averaging allows inferences to be based on the entire candidate model set as it uses information on each parameter proportional to the weight of the models providing parameter estimates (Anderson 2008). I used all the candidate models for model averaging, as poor models receive virtually no weight and therefore contribute very little to the model averaged result (Anderson 2008).

Are insects constant in their visitation patterns?

Various indices can be used to examine constancy in flower visitors. Bateman's Index measures the tendency of flower visitors to differentiate between floral morphs while controlling for preference (Waser 1986; Leebens-Mack & Milligan 1998). This index makes two assumptions; firstly, that all insects share the same inclination to distinguish between flower types, and secondly, that each flight between flowers is independent of previous flights (Leebens-Mack & Milligan 1998). To avoid violation of the first assumption, similar insects should be grouped together before the analysis is run. The second assumption is harder to correct, but violations can be avoided by calculating Bateman's Index for focal plants separated by increasing numbers of inter-plant flights (Leebens-Mack & Milligan 1998). However, Bateman's Index has other underlying problems (Chittka *et al*. 2001) because it cannot be calculated if flower visitors are completely constant to one species (as the denominator in the formula is zero). Moreover, if the frequency of inconstant movements from one species is zero, then the formula always returns maximum constancy, even if

flower visitors are inconstant when starting from the other species (Chittka *et al*. 2001). Therefore, I used a modified version of Bateman's Index (hereafter 'constancy index') that accounts for these underlying problems (Chittka *et al*. 2001) (Figure 3.4).

To avoid violating the assumptions associated with either index, I used the same four insect groups as for the preferences tests. I only calculated constancy index for movements between the first and second capitulum visited by the insects, because of the short foraging bouts undertaken by insects at my arrays, I rapidly ran out of replicates to calculate constancy for any further flights. The constancy index ranges from -1 (completely inconstant transitions), 0 (random transitions), through to +1 (completely constant transitions).

$$Bateman's\ Index = \frac{\sqrt{AxD} - \sqrt{BxC}}{\sqrt{AxD} + \sqrt{BxC}}$$

$$Constancy\ Index = 0.5\left[\frac{A-B}{A+B} + \frac{C-D}{C+D}\right]$$

		To	
		Species 1	Species 2
From	Species 1	A	B
	Species 2	C	D

Figure 3.4: Contingency table for calculating both Bateman's Index and the constancy index

Are insects just travelling to the closest capitulum?

If insects are not displaying constancy in their flights they should travel to the next closest flower to minimise travel costs (Chittka, Thompson & Waser 1999). To test for this with *Celmisia* flower visitors I looked at whether insects moved from the first capitulum in their foraging bout to the next closest capitulum. As *Celmisia* capitula were arranged in a 4 x 4 grid, I defined the next closest capitulum as any that were directly beside the capitulum (close) the insect had just left, including those on the diagonal. All capitula that were not directly beside the current capitulum were classed as non-neighbours (far). I set up a contingency table for the frequency of insect movements that were between the same or different species, and movements between close or far capitulum. I used chi-square tests of independence to test for associations between the type of *Celmisia* insects were flying

between and the distance insects were flying. All insect taxa were grouped together for this analysis and I ran the chi-square tests for each array type.

Results

Are Celmisia *species morphologically different?*

All floral characteristics I measured had some difference among *Celmisia* species (Table 3.2), but often this difference was driven by *C. lyallii* and *C. sessiliflora* (Figure 3.4).

Table 3.2: Results of the ANOVA tests on variation in *Celmisia* floral characteristics among species.

	df	Sum sq.	Mean sq.	F-value	p-value
Height					
Species	6	302297	503828	270	<0.001
Residuals	888	165705	1866		
Size					
Species	6	44052	7342	196.6	<0.001
Residuals	889	33196	37		
Disc					
Species	6	5162	860.3	370.9	<0.001
Residuals	889	2062	2.3		
Ray					
Species	6	4874	812.3	109.2	<0.001
Residuals	889	6615	7.4		
Ratio					
Species	6	19	3.167	26.34	<0.001
Residuals	889	106.8	0.120		

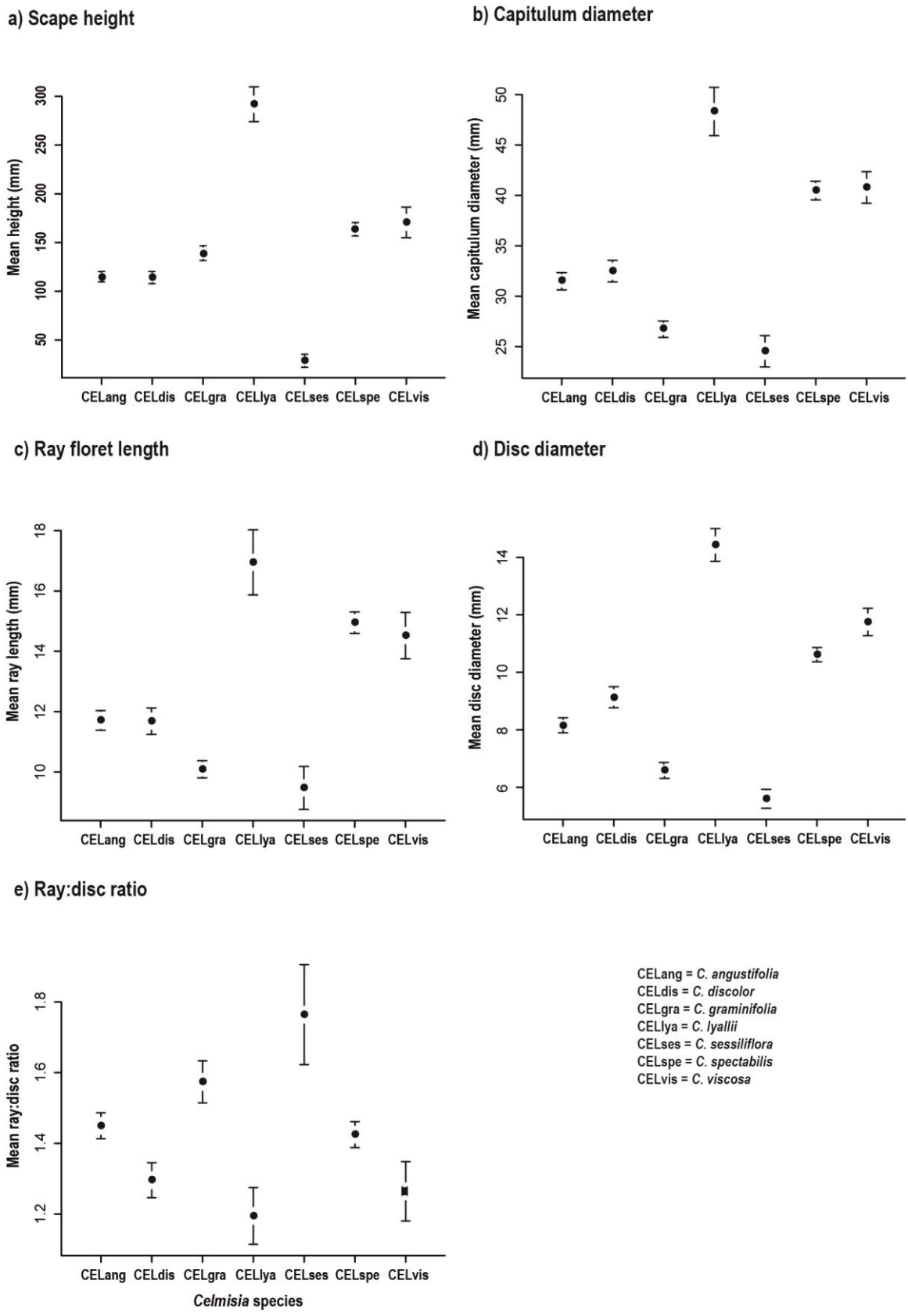

Figure 3.4: *Celmisia* floral traits. Dots represent the mean (± 95% CI) measurement (mm) for: a) scape height; b) capitulum diameter; c) ray floret length; d) disc diameter; e) ray:disc ratio.

The NMDS ordination confirmed that *C. lyallii* and *C. sessiliflora* were marginally different from the other *Celmisia* species across all floral characteristics, whereas the remaining five species were all morphologically more similar to each other (Figure 3.5).

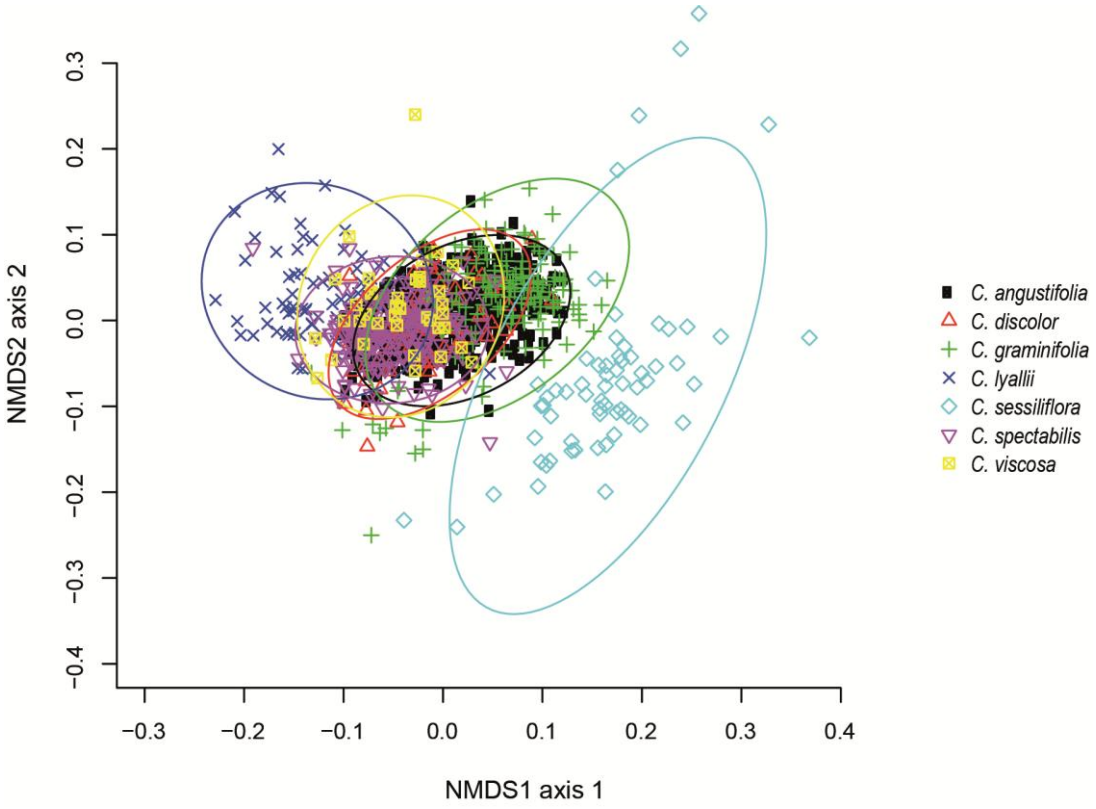

Figure 3.5: NMDS ordination showing the differences in floral traits between species. Each individual point represents an individual capitulum. The ellipses are the standard error around the mean of each group.

Do Celmisia *species share the same flower visitors?*

At arrays, *Celmisia* capitula were visited almost exclusively by flies (predominantly orders Empididae, Syrphidae, and Tachinidae) and bees (orders Apidae, Colletidae, and Halictidae). Two exotic insect species (*Bombus terrestris* and *Eristalis tenax*) visited the arrays. Feral honeybees (*Apis mellifera*) were observed at low elevation sites (1200-1300 m a.s.l.), but they never fed from capitula in arrays. Non-array based observations of *Celmisia* extend the list of flower visitors to include a few Coleoptera species, an extra Lepidoptera species and one observation of a bird (Table A2.1).

A NMDS ordination analysis on the frequency of visits by insect genera to seven *Celmisia* species showed *Celmisia* species share similar insect flower visitor communities (Figure 3.6). While there were some slight differences in the size of the centroid around each species, on the whole the *Celmisia* in this study tend to share insect flower visitors (Figure 3.6).

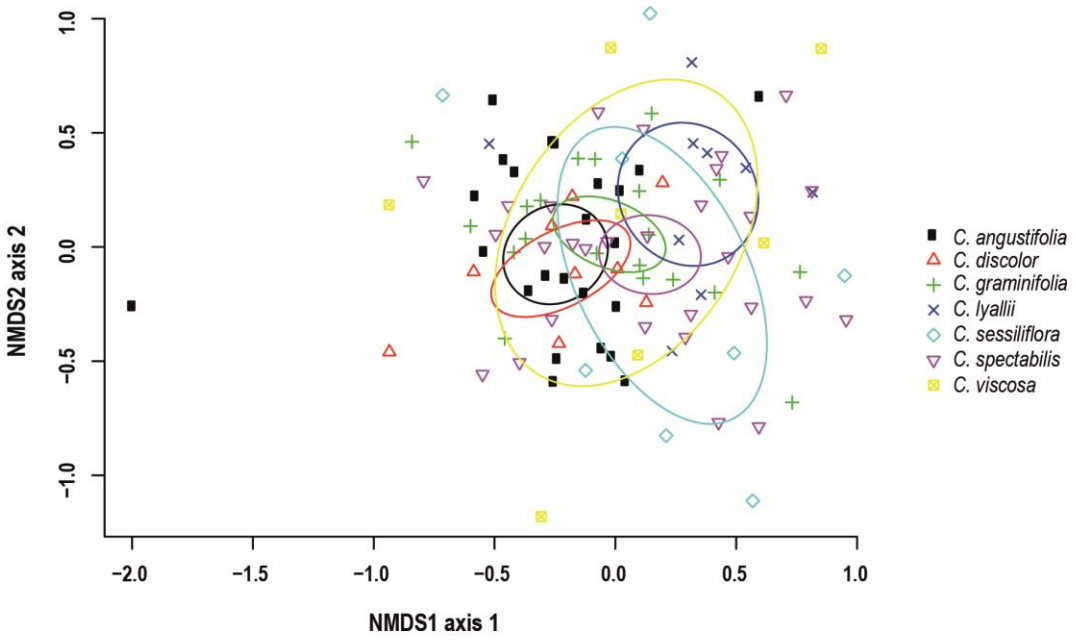

Figure 3.6: A NMDS ordination on the pollinator community of seven *Celmisia* species. Each point on the graph represents a *Celmisia* species at one array and the community of insects that were observed visiting it. The ellipses represent the standard error around the mean of each *Celmisia* species.

Generally insects were observed to visit all *Celmisia* species with only a few exceptions where an insect visited few or only one species (Figure 3.7).

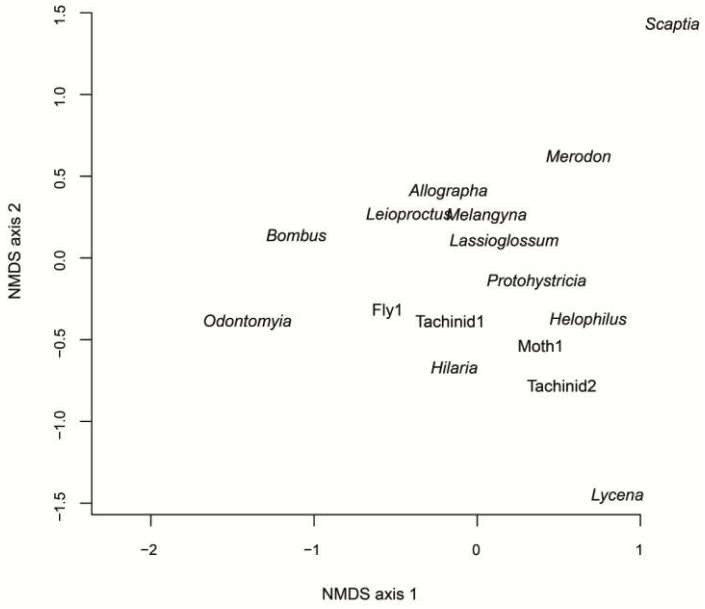

Figure 3.7: NMDS ordination showing the position of insect genera inside the ordination space.

A *Scaptia* fly was observed on only one occasion visiting *C. viscosa* and so is strongly associated with this species (Figure 3.6; Figure 3.7). Butterflies in the genus *Lycaena* were observed twice during array observations and only visited *C. sessiliflora* and *C. spectabilis*. *Odontomyia* insects tended to associated more closely with three of the *Celmisia* species (*C. angustifolia, C. discolor*, and *C. spectabilis*) (Figure 3.6; Figure 3.7).

Do insects show a preference for* Celmisia *species?

Altogether insect visitors to *Celmisia* showed a preference for one species over the other as the first species visited during their foraging bout at most of the array types (Table 3.3).

Table 3.3: Insect preferences to the first species visited during a foraging bout. Species in bold are those preferred by the insects.

Array Type	X^2	df	p
C. angustifolia : ***C. discolor***	9.41	1	0.002
C. angustifolia : *C. graminifolia*	28.73	1	<0.001
C. angustifolia : ***C. viscosa***	8.78	1	0.00
C. graminifolia : ***C. spectabilis***	19.15	1	<0.001
C. lyallii : *C. spectabilis*	1.62	1	0.203
C. sessiliflora : ***C. spectabilis***	112.78	1	<0.001

The proportion of insects that showed a preference to one species during their foraging bouts (Figure 3.8), almost exactly matched the preferences (or lack thereof) displayed by insects to the first species visited as they entered the array (Table 3.4); only the *C. angustifolia*: *C. discolor* array had a significant preference to first species visited become non-significant as the overall proportion of visits during a foraging bout.

Overall insect preference (Table 3.3) was driven by all insect taxa at one array (*C. sessiliflora*: *C. spectabilis*), two taxa at another array (*C. angustifolia*: *C. graminifolia*), or only one taxa (*C. graminifolia*: *C. spectabilis*) (Figure 3.8). The array with no overall preference (*C. lyallii*: *C. spectabilis*) resulted from the opposing preferences of two taxa balancing out (Figure 3.8).

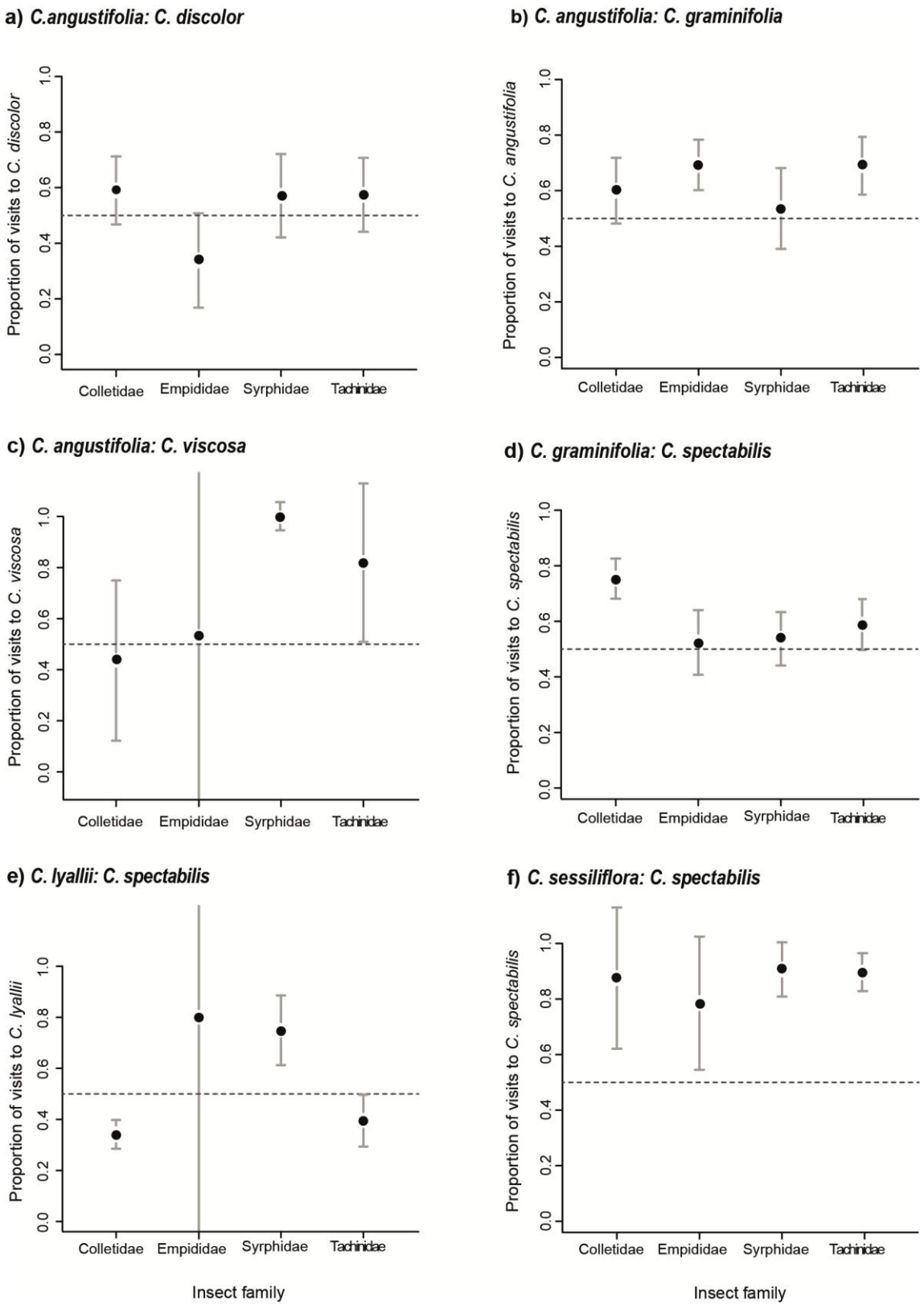

Figure 3.8: Insect preferences (mean ± 95% CI) shown here as a proportion of visits to one species at each array type by the four most common insect families. If the error bars do not overlap the line at 0.5 (the null hypothesis) then the insects show a significant preference for one of the two species. The overall preferred *Celmisia* species (from Table 3.3) is the one labelled on the y-axis.

Table 3.4: Tests for insect preference for the first species visited by the four most common insect families. Significant results are displayed in bold. For direction of preferences see Figure 3.8.

Array Type	Insect family	First Visit			Number of insects observed
		X^2	df	p	
C. angustifolia: *C. discolor*	Colletidae	6.15	1	**0.01**	47
	Empididae	0.05	1	0.83	21
	Syrphidae	1.98	1	0.16	41
	Tachinidae	1.59	1	0.21	51
C. angustifolia: *C. graminifolia*	Colletidae	2.2	1	0.14	55
	Empididae	27.74	1	**<0.001**	73
	Syrphidae	0.02	1	0.88	41
	Tachinidae	9.6	1	**0.002**	50
C. angustifolia: *C. viscosa*	Colletidae	0.36	1	0.55	25
	Empididae	1.8	1	0.17	5
	Syrphidae	4.57	1	**0.03**	37
	Tachinidae	2.57	1	0.12	14
C. graminifolia: *C. spectabilis*	Colletidae	50.90	1	**<0.001**	83
	Empididae	0.24	1	0.63	68
	Syrphidae	0.07	1	0.79	54
	Tachinidae	0.69	1	0.41	52
C. lyallii: *C. spectabilis*	Colletidae	0.67	1	0.41	6
	Empididae	4	1	0.05	4
	Syrphidae	5.33	1	**0.02**	48
	Tachinidae	0.75	1	0.39	65
C. sessiliflora: *C. spectabilis*	Colletidae	9.78	1	**0.002**	23
	Empididae	19.17	1	**<0.001**	23
	Syrphidae	34.78	1	**<0.001**	46
	Tachinidae	44	1	**<0.001**	56

Can floral characteristics predict the preference of insect visitors to* Celmisia*?

The intercept-only model was never included in the 95% confidence set, therefore providing some support that the models included within the 95% confidence set represented the data

well. All three arrays containing *C. angustifolia* had one model that was clearly the best model in the candidate set, in that it had a w_i >0.90 (Table 3.5). All three arrays containing *C. spectabilis* showed a high degree of model uncertainty as the top ranked model did not have a weight higher than 0.56 and several models were included in the 95% confidence set (Table 3.5).

Table 3.5: Results of model selection for each array type. Shown here are the 95% confidence sets for each array. H = scape height; R = ray:disc ratio; P = position in the array (edge/interior); Si = capitulum diameter; Se = capitulum sex (1,2,3 or 4); O = insect order (Coleoptera, Diptera, Hymenoptera, or Lepidoptera).

Array Type	Model	K[a]	log($\mathcal{L}$)[b]	AICc	Δi[c]	W_i[d]	Cum.w_i
C. angustifolia: *C. discolor*	H + R	4	0.98	138.1	0.00	0.98	0.98
C. angustifolia: *C. graminifolia*	P + H + Si + Se + O	9	-49.21	117.2	0.00	0.99	0.99
C. angustifolia:	H + Si	4	-7.94	24.6	0.00	0.94	0.94
C. viscosa	Si + Se	6	-8.79	31.2	6.57	0.03	0.97
C. graminifolia:	Si + Se	6	-17.98	48.27	0.00	0.56	0.56
C. spectabilis	Si + Se + O	7	-17.47	49.35	1.09	0.32	0.88
	P + Si + Se + O	8	-17.43	51.38	3.12	0.12	1.00
C. lyallii:	H + Se	6	-42.11	96.9	0.00	0.56	0.56
C. spectabilis	H + Se + O	8	-41.09	99.3	2.42	0.17	0.73
	H	3	-46.71	99.6	2.73	0.14	0.87
	H + R	4	-46.54	101.4	4.50	0.06	0.93
	P + H + R + Se + O	10	-40.34	102.4	5.52	0.04	0.97
C. sessiliflora:	H	3	-8.25	22.7	0.00	0.48	0.48
C. spectabilis	H + O	5	-6.56	23.6	0.89	0.31	0.79
	H + R	4	-8.13	24.6	1.88	0.19	0.98

[a] K - Total number of model parameters including the intercept and residual variance
[b] Log($\mathcal{L}$) – Log likelihood
[c] Δi - Difference between model AICc and minimum AICc value
[d] W_i - Probability of model *i* being the best in this set of candidate models

Height always appeared in the best model (expect for the *C. graminifolia*: *C. spectabilis* array where height could not be included in the candidate model set (models failed to converge; see Appendix 3)), suggesting that scape height is consistently an important component of insect floral preference across most of the species studied. This is most apparent in the array with the largest height difference between species (*C. sessiliflora*: *C. spectabilis*;

Figure 3.5), where insects almost never visited the shortest species (*C. sessiliflora*) first in their foraging bout (Table 3.4) or in subsequent visits to other capitula (Figure 3.8). Furthermore, the *C. sessiliflora*: *C. spectabilis* array was the only array type to have a top model with a single parameter (other than the random effect).

The sex of the capitulum also frequently occurred in the best model, or in the 95% confidence set for most array types; however, as the measure of floral sex was a measure of resource availability (pollen present/absent = male/female), it is not a particularly interesting measure of pollinator preference.

When the ray:disc ratio featured in the best model (*C. angustifolia*: *C. discolor*), the insects preferred to visit the species with a lower ray:disc ratio. As *Celmisia* species with a higher ray:disc ratio have larger ray florets (white) in comparison to disc florets (yellow), insect preferences for low ray:disc ratios indicate that insects prefer to visit yellower capitula. When the size of the capitulum appeared in the best model (*C. angustifolia: C. graminifolia; C. angustifolia: C. viscosa; C. graminifolia: C. spectabilis*), insects preferred to visit species with larger capitula.

The three arrays containing *C. spectabilis* had high model uncertainty and were also the only arrays known to have previously formed hybrids. In contrast, the three *C. angustifolia* arrays had a clear best model and no previous records of hybridisation. The *C. lyallii*: *C. spectabilis* array had the largest amount of model uncertainty (having five models in the 95% confidence set). In the *C. lyallii*: *C. spectabilis* array half the insect taxa preferred *C. lyallii* and the other half preferred *C. spectabilis*. It is therefore not surprising that there are no overall preferences to either *C. lyallii* or *C. spectabilis* (Figure 3.8), and thus also a high level of model uncertainty.

Are insects constant in their visitation patterns?

The constancy index showed that constancy was highly variable and depended both on insect family and array type (Table 3.6).

Table 3.6: Constancy index for flights between the first and second capitula visited in each array by four insect families. The constancy index produces values between -1 and +1, where: -1 = completely inconstant flights; 0 = completely random flights; and +1 = completely constant flights between plant species.

Array Type	Insect Family	Constancy Index	n
C. angustifolia: *C. discolor*	Colletidae	0.09	26
	Empididae	0.55	8
	Syrphidae	0.29	17
	Tachinidae	0.33	21
C. angustifolia: *C. graminifolia*	Colletidae	0.20	28
	Empididae	0.03	45
	Syrphidae	0.03	20
	Tachinidae	0.26	25
C. angustifolia: *C. viscosa*	Colletidae	0.43	10
	Empididae	0.50	3
	Syrphidae	1.00	10
	Tachinidae	1.00	4
C. graminifolia: *C. spectabilis*	Colletidae	0.23	35
	Empididae	0.12	32
	Syrphidae	0.01	29
	Tachinidae	0.17	28
C. lyallii: *C. spectabilis*	Colletidae	0.00	1
	Empididae	0.00	2
	Syrphidae	0.51	23
	Tachinidae	0.16	36
C. sessiliflora: *C. spectabilis*	Colletidae	0.50	8
	Empididae	0.00	9
	Syrphidae	0.92	13
	Tachinidae	0.43	31

No insect groups showed complete dissimilarity at any of the arrays (a score of -1); instead, two families were completely constant (a score of +1) at the *C. angustifolia*: *C. discolor* array, but most families exhibited close to random flight behaviour (a score of 0) (Table 3.6).

The constancy maps fairly well to the preferences shown by the insects (as a proportion of their foraging bouts), with insects that showed a higher level of preference were also being more constant in their foraging (Figure 3.9).

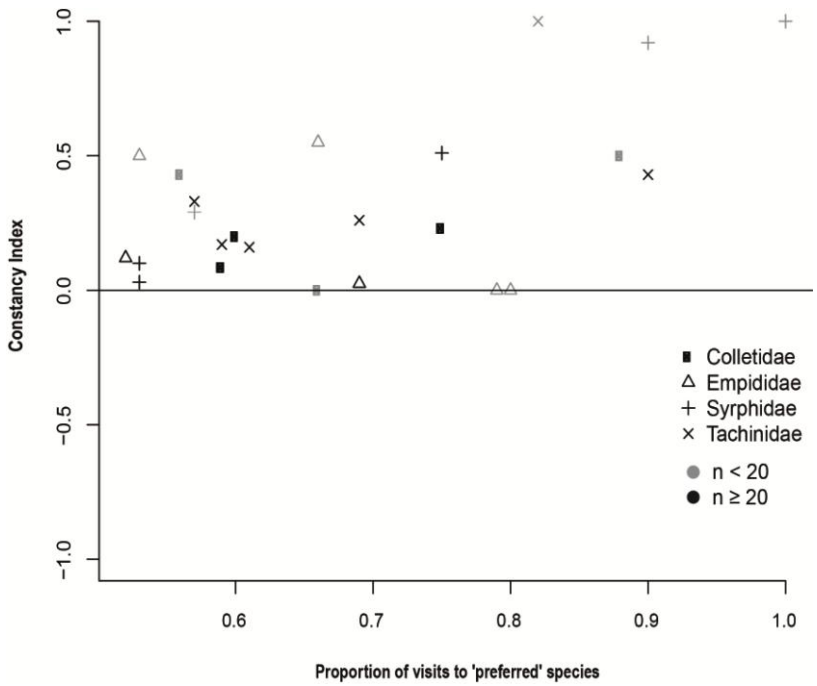

Figure 3.9: Preference and constancy of insect visitors to *Celmisia*. As the data points with the highest preference and constancy scores also had small sample sizes I did not test for correlation between preference and constancy.

Are insects just travelling to the closest capitulum?

The chi-square tests showed that most insect flights between the first and second capitula were not dependent on the location of the second capitulum (Table 3.7). The exception to this was the *C. angustifolia*: *C. graminifolia* array where insects moved further to continue foraging on the same *Celmisia* species (Table 3.7).

Table 3.7: Chi-square tests to see whether insect visitors move to the closest capitulum or to one further away. Significant p-values are shown in bold.

Array	Distance	Flights between species		X^2	df	p-value
		same	different			
C. angustifolia: *C. discolor*	near	25	26	0.23	1	0.63
	far	15	11			
C. angustifolia: *C. graminifolia*	near	35	40	4.91	1	**0.03**
	far	33	15			
C. angustifolia: *C. viscosa*	near	46	44	0.15	1	0.69
	far	34	27			
C. graminifolia: *C. spectabilis*	near	19	11	0.01	1	0.93
	far	28	15			
C. lyallii: *C. spectabilis*	near	29	6	0.51	1	0.48
	far	32	3			
C. sessiliflora: *C. spectabilis*	near	16	6	0.40	1	0.53
	far	9	1			

Discussion

All the *Celmisia* species capitula observed in my study shared the same insect pollinator community, but since pollinator behaviour is more important than the make-up of the pollinator community (Esfeld *et al*. 2009), the community alone is not an accurate measure of hybridisation potential. Despite the almost overwhelming similarities between *Celmisia* species in my study (see cover picture for this chapter), insects displayed preferences for some *Celmisia* species over others. To some extent these preferences also translated into constancy, as although constancy values were low, no insect family showed any dissimilarity in their foraging bouts.

I consistently found scape height to be an important predictor of which species an insect would visit as it entered the array. In all but one case (*C. lyallii*: *C. spectabilis* array) this finding corresponded to the overall preference shown by insects to the first species visited. Height was still an important predictor in the *C. lyallii*: *C. spectabilis* arrays as insects also preferred taller capitula within a species. Taller plants are known to attract more insect flower visitors than their nearest neighbours (Donnelly, Lortie & Aarssen 1998; Carromero & Hamrick 2005; Young 2006; Esfeld *et al*. 2009).

Pollinators generally prefer to visit larger floral displays than smaller ones (Ohashi & Yahara 2001). In my study, when capitulum size was included in the top models, insects always preferred the larger species. If pollinators prefer the taller species (perhaps because they are easier to see above the grass (Gumbert & Kunze 1999)), and those with larger capitula, then *C. sessiliflora* appears to have the worst of both worlds. *Celmisia sessiliflora* has one of the smallest of all *Celmisia* capitula and is also one of the shortest (Fenner, Lee & Pinn 2001), and in my study received the lowest rate of insect visitation.

Constancy in generalist pollinator communities

Waser (1998) suggested that generalisation in pollinating insects would make strong floral isolation during and after speciation highly unlikely. However, a few studies have since made a case for pollinator-mediated reproductive isolation in generalist pollinator communities (Yang, Gituru & Guo 2007; Marques *et al*. 2012). Jones & Reithel (2001) found that interspecific flights occurred more often between species with similar flower colours, and in their case the flowers were also yellow and white. Bees are known to be less constant to flowers with simple morphology (Chittka, Thompson & Waser 1999), while syrphids are generally thought not to have strong preferences (Branquart & Hemptinne 2000). Similar ideas have dominated the discussion of New Zealand alpine pollinator behaviour; therefore are New Zealand insects likely to be good barriers to reproductive isolation?

I observed a much smaller subset of insect flower visitors than other recent studies working in nearby sub-alpine habitats (Young 2006; Sciligo 2009). Young (2006) looked at the insect visitors to *Aciphylla* species which have more visible inflorescences than *Celmisia* as they are very tall, but Sciligo (2009) studied insect flower visitors to several *Drosera* species, which are tiny in comparison to *Celmisia* and do not suggest an obvious reward to pollinators. It is likely that working at higher altitudes resulted in a subset of the pollinating fauna that Young (2006) (sites ranged between 700 to 900 m a.s.l.) and Sciligo (2009) (study site ~900 m a.s.l.) found at lower elevations (Arroyo, Primack & Armesto 1982).

Due to the short time frame in which each array observation took place, it is likely that some insect groups were under represented. For example, beetles are less mobile than other insect groups (Newstrom & Robertson 2005), therefore array experiments may not have run long enough for beetles to find them. Additionally, beetles that I observed on *Celmisia* were very easily startled, so perhaps I did not get beetle visitation at arrays because of my presence nearby. Furthermore, many studies discount beetles as pollinators because they are expected to carry little pollen and do not often move far (Newstrom & Robertson 2005). However, beetles can be extremely abundant on mat daisies in the New Zealand high country (Newstrom & Robertson 2005), and I observed large numbers of

Curculionidae beetles on *C. sessiliflora* mats while surveying my phenology transects (Chapter 2). Night surveys were not undertaken due to the difficulty of such work alone in the alpine environment. However, I observed that although some *Celmisia* species might close their capitula over night, the potential role of night pollinators cannot be ruled out and could provide an interesting avenue for further study.

Recent studies by Campbell *et al*. (2010; 2012) have made a strong case for New Zealand alpine flower-visiting insects being less generalist than previously thought. Campbell *et al*. (2010) found syrphid flies and *Leioproctus* bees to have a strong preference for yellow over white coloured flowers, and the results of a factorial experiment suggested that insects could be responding to a variety of floral characteristics. Additionally, Campbell *et al*. (2010) stated that even minor morphological differences between species could influence the visitation of New Zealand alpine insects. Campbell *et al*. (2012) provided further support for these findings through a series of experiments manipulating floral colours in *Wahlenbergia albomarginata*, a common alpine herb.

My findings of insect preferences for some *Celmisia* species over others are all consistent with these findings by Campbell *et al*. (2010; 2012). Furthermore, I have shown that some New Zealand alpine flower visitors are influenced by scape height and capitulum size as well as the differences in colour and floral shape as found by Campbell *et al*. (2010). Insect preferences did not completely translate to constancy which has also been found before in a study on butterflies in Colorado (Pohl, Van Wyk & Campbell 2011). Perhaps this is not surprising in *Celmisia* due to the large morphological similarities between species.

Floral traits and Constancy

Constancy arises from a pollinator's limited memory for multiple floral characteristics (Chittka, Thompson & Waser 1999), therefore bypassing some flowers is the best strategy if increased travel costs are offset by reduced handling time (Waser 1986). Pollinators that are able to learn how to handle several flower types should be less constant (Waser 1986), therefore the more dissimilar species become, the higher the level of insect pollinator constancy (Waser 1986; Gegear & Laverty 2001). Plants with different floral syndromes epitomise this theory. For example, Kay & Schemske (2003) found that plants with different floral syndromes had little or no overlap in pollinator visitation, but plants sharing a pollinator syndrome also shared pollinators.

Floral colour is thought to be important for determining constancy in foraging insects and most studies of insect constancy are on species that primarily differ in colour (Gegear & Laverty 2001). Interspecific flights by insects occur more often between species with similar flower colours (Chittka, Thompson & Waser 2001; Jones & Reithel 2001), but similar looking species in general will lead to a lack of constancy in insects (Chittka, Gumbert & Kunze

1997). Yet, many flowering communities do not have large differences in floral colours (Gegear & Laverty 2001).

If insects are not displaying constancy, then optimal foraging theory dictates that they move to the closest flowers in order to minimise travel associated costs (Chittka, Thompson & Waser 1999). Higher assortative mating is more likely when species are clumped together with conspecifics (Jones & Reithel 2001; Hershl & Roy 2007), especially if the clump contain high rewards (Esfeld *et al*. 2009). Most pollinators lie somewhere between the two extremes (high travel cost – visit nearest neighbour; high handling cost – be constant) (Gumbert & Kunze 1999). I only found evidence of insects visiting the closest capitulum in one of the six array types, with most movements by insects being independent of distance. It is possible that my experiment was on too small a scale for insects to incur any travel related costs as they moved between capitula, but insects were also not displaying high levels of constancy, so perhaps *Celmisia* are not different enough to drive higher handling costs for insect visitors. Although floral traits can predict overall insect preferences to the first capitulum visited at most arrays, this does not translate into constancy for insect flower visitors to *Celmisia*.

Conclusions

Chittka, Thompson & Waser (1999) suggested it was unlikely pollinators were so constant they could lead to complete or nearly complete reproductive isolation. In contrast, Aldridge & Campbell (2007) saw pollinator-mediated reproductive isolation as the key isolating mechanism between two species, but noted that it was rarely complete. Pollinator communities are highly stochastic as they are influenced by a variety of abiotic and biotic factors other than the presence of flowering species (Yang, Gituru & Guo 2007). Therefore it is likely that the evolution of multiple barriers will occur if hybrids really are exerting a negative fitness cost on either parent.

The seven *Celmisia* species in my study all shared similar insect flower visitors. Some insect families did show preferences to some *Celmisia* species, especially at arrays with *Celmisia* species pairs that do not form natural hybrids. Although insect preferences did not always render strong constancy during an insect's foraging bout, these preferences alone may be enough to cause some reproductive isolation in *Celmisia,* but it is in no way complete.

A limitation of my study is that I do not know whether floral visitation also means pollination. Further work could be done here to determine whether insects are moving pollen between different species of *Celmisia*. Pollen dyes would make this work less challenging as *Celmisia* pollen grains are all extremely similar to each other (Moar 1993). Non-random visitation is a first step towards showing non-random pollen transfer (Jones 1997), and

Campbell *et al*. (2010) suggested that visitation was likely to lead to pollination in their study of New Zealand alpine flower visitors, however they also stressed that more work was needed on this topic.

Experimental tests of insect behaviour in natural environments are essential for teasing out the role of floral visitors in reproductive isolation (Pohl, Van Wyk & Campbell 2011). Prentis *et al*. (2007) suggested that when species had little habitat differentiation, substantial flowering overlaps, and were pollinated by the same insects, high rates of hybridisation were not unexpected. However, in their study on *Senecio*, they found that mature hybrids were completely absent from sympatric populations thus indicating a role for postzygotic isolating barriers in their system. My work suggests that if pollinator-mediated reproductive isolation exists in *Celmisia* it is at low levels and is a weak barrier to hybridisation, meaning alternative barriers must be responsible for the observed paucity of hybrids in this genus.

CHAPTER FOUR

How successful are *Celmisia* hybrids after their formation?

A flowering *Celmisia viscosa* hybrid.

Introduction

Postzygotic reproductive isolating barriers are generally considered to be less effective than prezygotic barriers (Ramsey, Bradshaw & Schemske 2003; Kay 2006; Martin & Willis 2007; Lowry *et al.* 2008). However, if postzygotic barriers block what other barriers miss, they could still play an important role in reproductive isolation as even tiny amounts of gene flow can lead to introgression (Widmer, Lexer & Cozzolino 2009). Some postzygotic isolating barriers are extremely effective at preventing hybrid formation and growth, for example, Barros *et al*. (2007) found no viable hybrid seeds after performing experimental hand-crosses. Furthermore, hybrid sterility is thought to be a major cause of postzygotic reproductive isolation (Bomblies 2010).

Many studies have recorded fertile hybrids (Mallet 2008), but environmental factors (extrinsic reproductive isolation) could also contribute to the success or otherwise of hybrids (Coyne & Orr 2004). Extrinsic reproductive isolation is generally considered a less effective barrier than intrinsic reproductive isolation (Lowry *et al*. 2008), as intrinsic effects can have strong impacts resulting in little or no hybrid production (e.g. Barros *et al*. 2007). However, interactions between hybrids and other organisms can have a substantial impact on the success of a hybrid (Campbell *et al*. 2002). For example, natural enemies such as pre-dispersal seed predators can have severe negative effects on hybrids leading to outbreeding depression in the hybrid (Strauss 1994). Interactions between a hybrid and other organisms can be complex; for example, hybrids may harbour higher levels of pests than their parents, thereby functioning as a sink, drawing pests away from the parent species (Witham 1989). Alternatively, hybrids could expand the host range of pests and pathogens by acting as a bridge between parent species (Strauss 1994).

Hybrid fitness can break down in later generations, so that F_1 hybrids with strong heterosis (hybrid fitness) do not necessarily produce equally fit offspring (Campbell *et al.* 2008). Most studies do not examine the success of later generation hybrids, due to the longer time scales involved, particularly in perennial plant species (Kirk, Vrieling & Klinkhamer 2005). Hybrid breakdown as a result of environmental factors is not consistent, as abiotic factors are highly variable so what favours hybrids at one point in time may be a disadvantage later (Coyne & Orr 2004).

Pre-dispersal seed predation

Selective pre-dispersal seed predation can function as a reproductive isolating barrier by reducing the number of viable hybrid seeds available for dispersal (Cummings, Alexander & Snow 1999). Seeds are a readily available, highly nutritious source of food, especially in comparison to vegetative material in plants (Fenner & Thompson 2005). Losses of seeds to

pre-dispersal predators can be greater than 50% of the plant's seed crop (Boierio *et al*. 2010), and are often up to 90% of the seed crop (Fenner & Thompson 2005; Honek & Martinkova 2005), severely reducing the number of viable seeds (Weppler & Stocklin 2006). Additionally, seed predators can cause indirect damage to seeds they do not feed on (De Menezes *et al*. 2010). Most seed predators are larvae of small, specialised insects belonging to the orders; Coleoptera, Diptera, Hemiptera, Hymenoptera, and Lepidoptera (Crawley 1992; De Menezes *et al.* 2010), and their effects can range from damage to individual plants through to altering the composition of plant communities (Kolb, Ehrlel & Eriksson 2007).

Seed predators are well known in *Celmisia* (Dugdale 1974; Molloy 1975; Spence 1990), and previous records include findings of Diptera, Coleoptera, Lepidoptera (Dugdale 1974), and Hemiptera (Larivigre 2002) inside the seed-heads (Burrows 1961). While Diptera have been previously recorded in a Celmisia hybrid (*C. x pseudolyallii*) (Molloy 1975), it is not known whether the hybrid harbours higher numbers of predators than either of its parent species.

Germination of hybrid seeds

If seeds survive pre-dispersal predation, failure to germinate can also function as a postzygotic isolating barrier in plant species. Hybrids are often thought to have lower germination or higher rates of seedling mortality than their parents (Ackerman, Achatz & Weigend 2008). Intrinsic postzygotic reproductive isolation could be important here if divergent genomes result in developmental incompatibles (Rieseberg 2001; Lowry *et al.* 2008).

Extrinsic reproductive isolation could also be important if seed germination is influenced by environmental factors such as weather conditions or soil type (Campbell & Waser 2007). *Celmisia* are known to produce a low proportion of filled seed (probably as a result of seed predation), but generally germinate readily (Scott 1975). Detailed instructions exist on the cultivation of *Celmisia* (Cartman 1985; Metcalf 1993), and one experimental study investigated various factors that might facilitate germination (Scott 1975). It is not known whether *Celmisia* hybrids produce fewer seedlings than their parent species.

I investigated the effects of seed predators and germination as potential postzygotic reproductive isolating barriers in natural *Celmisia* hybrids found at Craigieburn Valley Ski Area. I asked:

1. Do *Celmisia* hybrids have greater rates of seed predation than either parent species?
2. Do *Celmisia* hybrids have different kinds of seed predators than their parents?
3. Do *Celmisia* hybrids have lower germination success than either parent species?

Methods

Field methods

I used the naturally occurring *C. lyallii* X *C. spectabilis* hybrid (*C. x pseudolyallii*) for both the seed predator study and germination experiment. *Celmisia x pseudolyallii* occurs wherever the two parent species are found together (Metcalf 1993), and is often found in disturbed habitats (Given 1984). Other *Celmisia* hybrids are also present at Craigieburn Valley Ski Area, and as they were also producing seed at the time of my study I included them in the seed germination experiment. These other hybrids were: *C. lyallii* X *C. sessiliflora* and *C. viscosa* X an unidentified parent (hereafter referred to as *C.* sp.).

Pre-dispersal seed predation

I collected *Celmisia* seed-heads from *C. lyallii*, *C. spectabilis* and their hybrid *C. xpseudolyallii* at an early stage of seed development from both basins at Craigieburn Valley Ski Area in February 2012. *Celmisia x pseudolyallii* is the most common *Celmisia* hybrid at Craigieburn Valley Ski Area (*pers. obs.*). During seed collection I located hybrid plants bearing seed-heads and collected seed-heads at a similar stage of development on the closest plants of each parent species. I assumed that the closest parent plants were most likely to be the true parents of each hybrid as *Celmisia* seeds rarely disperse far from the parent plant (Spence 1990). Gathering seed-heads from the closest parent plants to each hybrid also controlled for spatial variation in seed predator density. Collecting seed-heads in this manner resulted in a distinct spatial structure to my data, therefore one hybrid and both of its parents formed one replicate/block in later analysis.

Seed-heads were placed into small plastic containers with lids allowing air flow and left at room temperature but out of direct sunlight until insects began to emerge from the seed-heads. Insects were counted out into their respective orders and the Diptera, Hemiptera, and Hymenoptera were frozen. The Lepidoptera were left to pupate inside the containers and are currently still in the process of pupating.

Germination of hybrid seeds

To determine whether a lower proportion of hybrid seeds germinated in comparison to their parent species across three natural *Celmisia* hybrid types found at Craigieburn Valley Ski Area (Table 4.1). I used the collection structure as described for the seed predation work above.

Table 4.1: *Celmisia* hybrid combinations, with the number of replicates (blocks) and seeds planted for each hybrid type. Fewer replicates for the *C. lyallii* X *C. sessiliflora* and the *C. viscosa* + hybrid experiments reflects the natural abundance in the field.

Hybrid type	Number of replicates	Number of seeds per replicate
C. lyallii X *C. spectabilis*	30	30
C. lyallii X *C. sessiliflora*	6	7, 8, 15, 15, 15, 15
C. viscosa X *C.* sp.	4	3, 30, 30, 30

Seeds were collected directly from the seed head in the field as they became ripe (February-March 2011). All seeds were stored in paper envelopes and then frozen for approximately one month until they were planted out into glasshouse trays. Seeds were sown into a potting mix containing one third each of peat, sand, and fine bark. A fertiliser mix containing 139.2 g of super phosphate and 69.6 g of dolomite lime per 120 litres was also added to the potting mix. Each block remained in one tray, and seeds were evenly placed on top of the potting mix. A thin layer of fine stone chip was placed on top (following Metcalf 1993).

Figure 4.1: From left to right; *Celmisia* seeds placed out into the tray, after the stone chip has been placed on top, a new *Celmisia* cotyledon (with a toothpick for scale).

Although germination experiments in the field would provide more realistic germination and growing conditions (Martin & Willis 2007), with small numbers of seed it was important to maximise the number of seedlings observed, so the trays were placed into an alpine glasshouse at the University of Canterbury campus in Christchurch, New Zealand. Trays were shaded from direct bright light (following Metcalf 1993), and were watered by an automatic watering systems three times a week.

I monitored the *Celmisia* germination monthly from the seed sowing date. I recorded the number of new cotyledons appearing each month and marked individual plants with a toothpick to avoid recounting them the following month. I randomly rearranged the trays every other month so that positioning of the tray would not affect the germination of some trays more than others. I stopped monitoring the experiment after the number of new

Celmisia seedlings had stopped germinating at around eight to ten months depending on the *Celmisia* taxon.

Data analysis

All statistical tests were performed in the programme 'R' version 2.14.1 (R Core Development Team 2012). The specific tests for each experiment are outlined below.

Pre-dispersal seed predation

To see whether hybrids had greater rates of seed predation than either parent species, I used a Poisson generalised linear model with the number of insects per capitulum as the response and *Celmisia* taxa as the predictor. I included a blocking factor (the *Celmisia* hybrid plus parent group) in order to account for the spatial variation in the data set. As is normal with a blocking factor I did not include an interaction term as I was not directly concerned in whether the number of seed predators found inside *Celmisia* seed heads depended on the blocking factor. Additionally, while the seed-heads were collected across a range of altitudes, the majority were from lower altitudes, so any effect of altitude would not have been apparent.

To test whether the hybrid had variation in the types of insect orders in comparison to the parent species, I used a Poisson generalised linear mixed effects model (GLMM) with block as the random effect and *Celmisia* taxa and insect order as the fixed effects using the 'lme4' package in R (Bates, Maechler & Bolker 2011). I included an interaction term between the fixed effects to determine whether the number of different seed predators depended on the *Celmisia* taxa.

Germination of hybrid seeds

To determine whether hybrids suffered germination failure (in comparison to their parent species) I used a binomial generalised linear model with the proportion of seeds planted that germinated as the response and *Celmisia* taxa as the predictor. In order to account for the spatial variation in the data I included a blocking factor (the *Celmisia* hybrid plus parents group). There was evidence for overdispersion in the *C. lyallii* X *C. spectabilis* hybrid data, so I re-ran the model using quasibinomial errors. Again I did not include an interaction term between the type of *Celmisia* and the blocking factor.

Results

Pre-dispersal seed predation

I found Diptera, Lepidoptera, Hemiptera, and Hymenoptera inside capitula of *C. lyallii*, C*. spectabilis* and their hybrid. All Diptera were in the genus *Trupanea* (family: Tephritidae),

while the Lepidoptera were probably in the family Geometridae (based on previous records (Molloy 1975; White 2002): note I am still waiting for my collected specimens to complete their pupation). The Hemiptera were all seed bugs in the genus *Rhypodes* (family: Lagaeidae). The tiny Hymenoptera were presumed to be parasitoids of the other insects.

The overall number of insects per capitulum in the hybrid was not significantly different to the number of insects per capitulum found inside either parent (Table 4.2; Figure 4.2).

Table 4.2: ANOVA table from the seed predation Poisson Generalised Linear Model

	df	Deviance	Residual df	Residual deviance	p-value
NULL			92	158.26	
Block	30	65.121	62	93.14	0.0002
Celmisia species	2	4.300	60	88.84	0.12

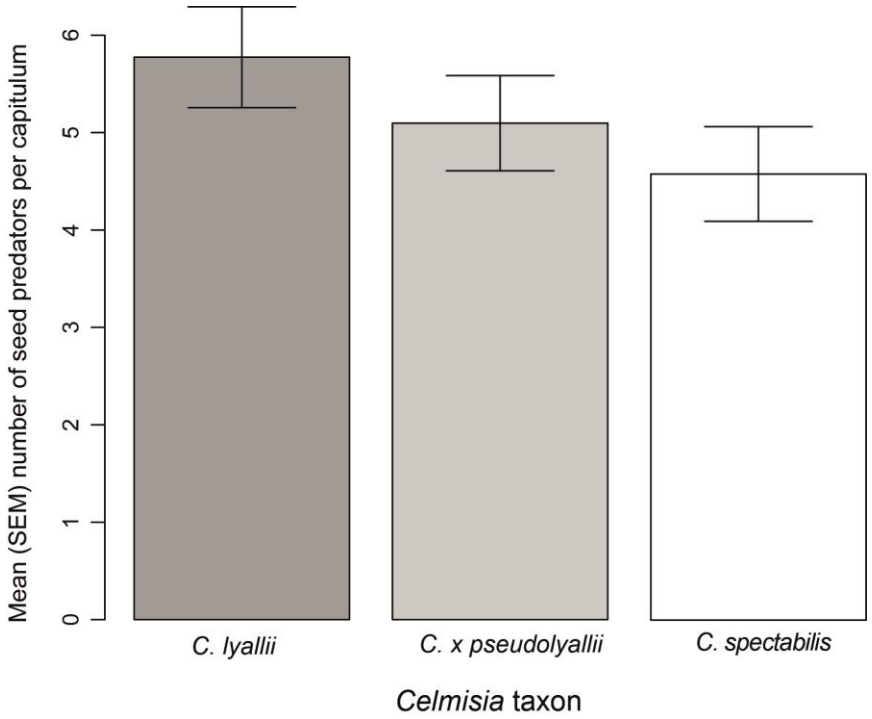

Figure 4.2: Mean (±SEM) number of seed predator individuals (all species combined) per inside *Celmisia* seed-head.

There were however differences in the species composition of the seed predators. There were significantly fewer *Trupanea* flies in *C. spectabilis* than in either *C. lyallii* or *C. x pseudolyallii*, and there were significantly fewer *Rhypodes* bugs in the hybrid than in either *C. lyallii* or *C. spectabilis* (Table 4.3; Figure 4.3). There were more Lepidoptera in *C. lyalli*

than either of the other two Celmisia taxon, but due to small sample sizes this difference was not significant (Table 4.3; Figure 4.3).

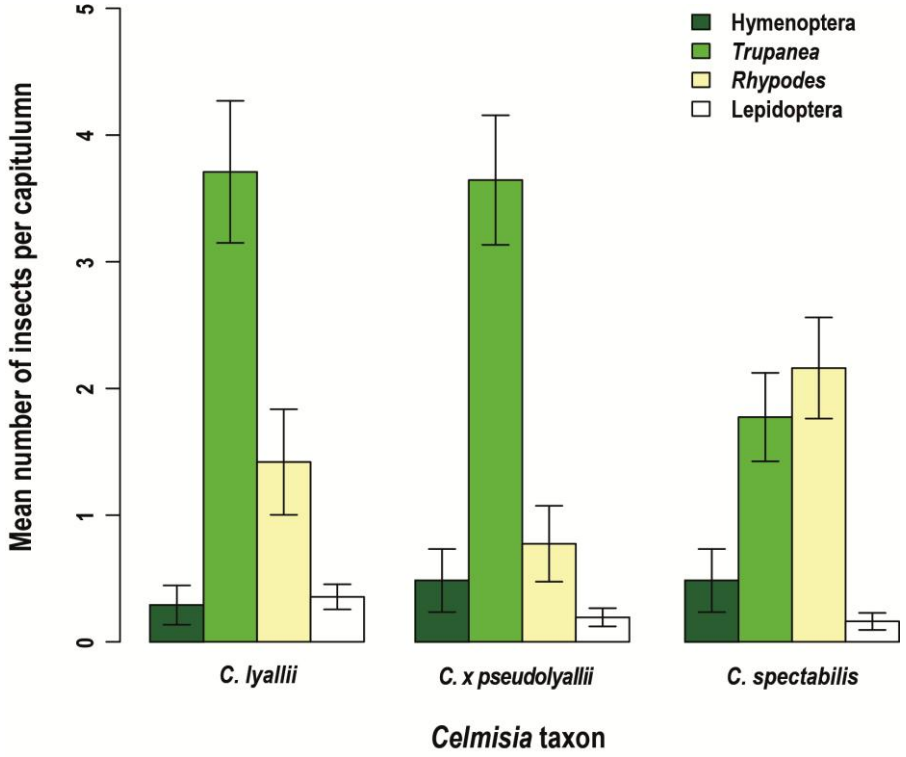

Figure 4.3: Mean (± SEM) number of four insect orders found inside the seed-heads of *C. lyallii*, *C. spectabilis* and their hybrid *C. x pseudolyallii*.

Table 4.3: Estimates and standard errors of fixed effects from the model: number of seed predators ~ insect order**Celmisia* type + (1|Block). Significant effects and interactions are indicated in bold.

Parameter	Estimate	Std. error	z value	p value
Intercept	**-1.27**	**0.34**	**-3.76**	**<0.001**
C. spectabilis	0.51	0.42	1.21	0.228
C. x pseudolyallii	0.51	0.42	1.21	0.228
Trupanea	**2.55**	**0.35**	**7.33**	**<0.001**
Rhypodes	**1.59**	**0.37**	**4.32**	**<0.001**
Lepidoptera	0.20	0.45	0.45	0.657
C. *spectabilis*: *Trupanea*	**-1.25**	**0.45**	**-2.75**	**0.006**
C. x pseudolyalii: *Trupanea*	-0.53	0.44	-1.19	0.234
C.spectabilis: *Rhypodes*	-0.09	0.47	-0.19	0.846
C. x pseudolyallii*: *Rhypodes	**-1.12**	**0.49**	**-2.26**	**0.024**
C. spectabilis: Lepidoptera	-1.30	0.69	-1.89	0.059
C. x pseudolyallii: Lepidoptera	-1.12	0.66	-1.69	0.092

Germination of hybrid seeds

Across all species germination at the end of the experiment was modest (Figure 4.4), with only 29.6%, 35.6% and 27.4% of all seeds planted germinating in the *C. lyallii* X *C. spectabilis*, *C. lyallii* X *C. sessiliflora*, and *C. viscosa* X *C.* sp. combinations respectively. Germination varied across the species, with *C. viscosa* and its hybrid having the lowest and highest percentage of seeds to germinate respectively at the end of the experiment (Table 4.4).

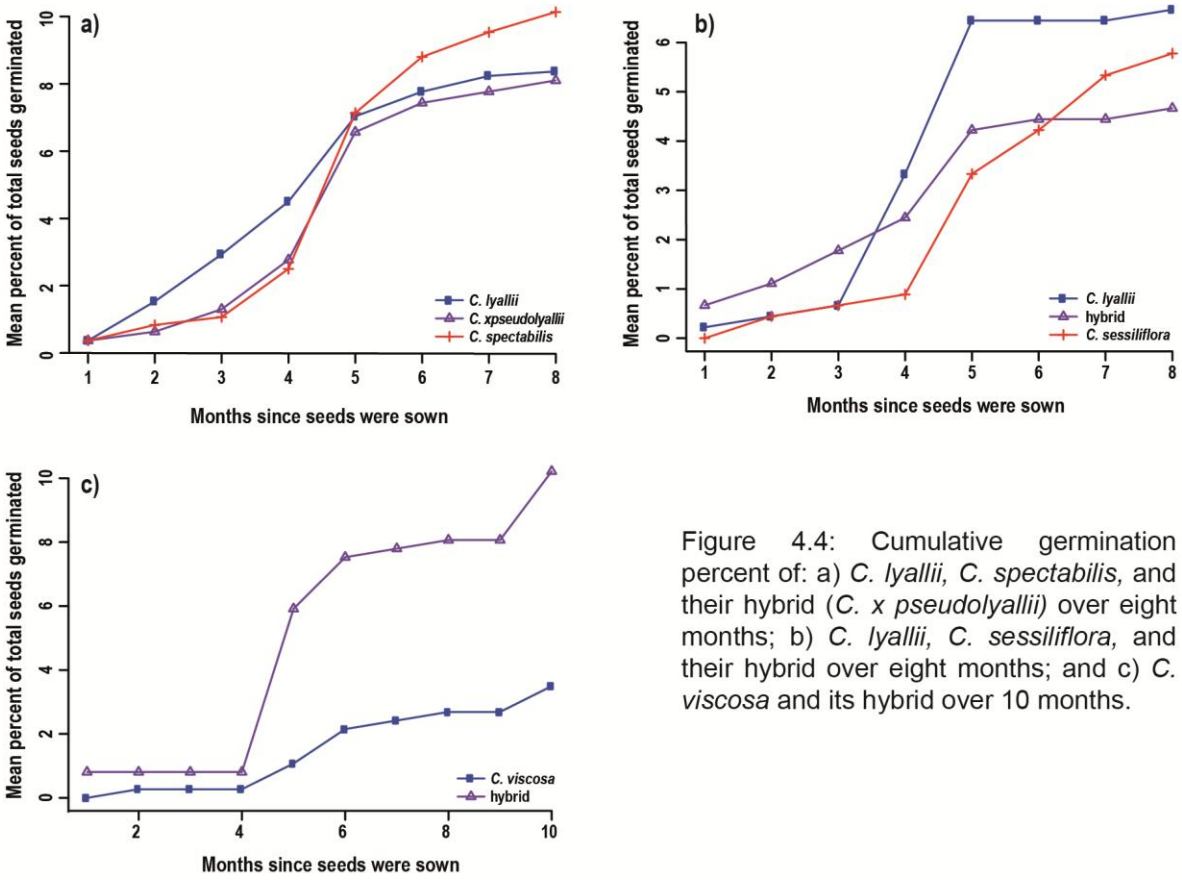

Figure 4.4: Cumulative germination percent of: a) *C. lyallii, C. spectabilis,* and their hybrid (*C. x pseudolyallii)* over eight months; b) *C. lyallii, C. sessiliflora,* and their hybrid over eight months; and c) *C. viscosa* and its hybrid over 10 months.

Table 4.4: Percent of seeds to germinate per *Celmisia* taxon at the end of the experiment.

Hybrid Type	*Celmisia* taxon	Percent germinated
C. lyallii X *C. spectabilis*	*C. lyallii*	28
	C. spectabilis	34
	C. x pseudolyallii	27
C. lyallii X *C. sessiliflora*	*C. lyallii*	40
	C. sessiliflora	37
	hybrid	29
C. viscosa X *C.* sp.	*C. viscosa*	14
	hybrid	41

Eight months after the seeds were sown there was no significant difference in the proportion of seeds that germinated between hybrid and parent seedlings for either the *C. lyallii* X *C. spectabilis* or *C. lyallii* X *C. sessiliflora* combinations (Figure 4.5a,b; Table 4.4). Conversely, in the *C. viscosa* X *C.* sp. combination there were significantly more hybrid seedlings than *C. viscosa* seedlings at 10 months after the seeds were sown (Figure 4.5c; Table 4.5). Hence, there was no indication in any of the hybrids that germination was worse than the putative parents.

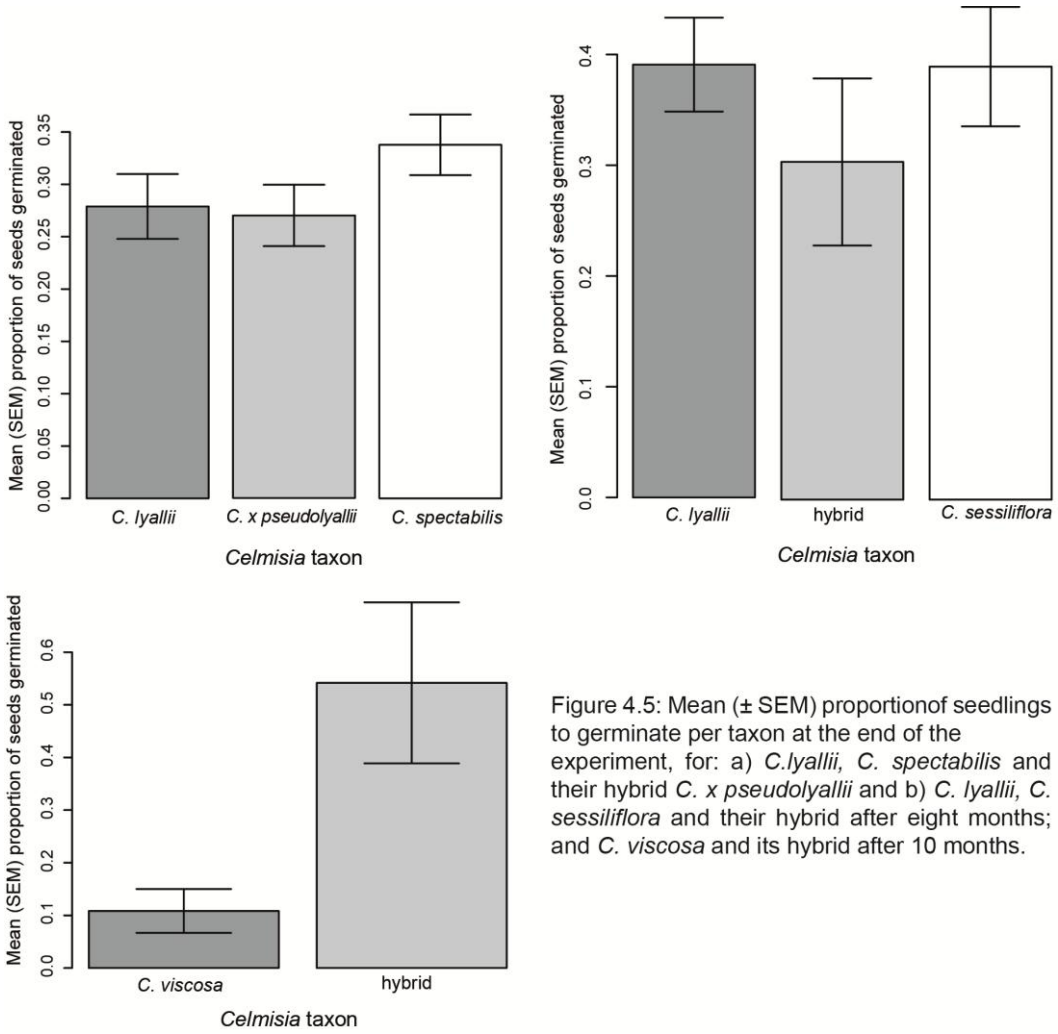

Figure 4.5: Mean (± SEM) proportionof seedlings to germinate per taxon at the end of the experiment, for: a) *C.lyallii, C. spectabilis* and their hybrid *C. x pseudolyallii* and b) *C. lyallii, C. sessiliflora* and their hybrid after eight months; and *C. viscosa* and its hybrid after 10 months.

Table 4.5: Results of binomial generalised linear models on the proportion of hybrid and parent *Celmisia* seeds to germinate.

Hybrid Type		df	deviance	residual df	residual deviance	F - value	p-value
C. lyallii x	NULL			89	391.03		
C. spectabilis	species	2	11.60	87	379.43	1.64	0.20
	block	29	149.32	58	230.11	1.45	0.11
C. lyallii x	NULL			17	18.58		
C. sessiliflora	species	2	2.04	15	16.54		0.36
	block	5	9.54	10	6.99		0.09
C. viscosa x	NULL			7	25.23		
C. sp.	species	1	17.46	6	7.77		<0.001
	block	3	1.77	3	5.99		0.62

Discussion

Pre-dispersal seed predation

Overall, the hybrid *C. x pseudolyallii* had the same number of larvae per capitulum as both its parent species; however, there were differences in the type of insects found inside *Celmisia* seed heads. My finding of fewer *Trupanea* flies inside *C. spectabilis* than in *C. lyallii* is consistent with work by Spence (1990). The hybrid also had more *Trupanea* than *C. spectabilis* but not *C. lyallii*; however, it had fewer *Rhypodes* bugs than either parent. Therefore, if different insect groups exert different levels of damage to seeds, there could still be a route for weak reproductive isolation in *Celmisia* via seed predators. As Hemiptera feed on seeds by piercing the seed coat, the amount of damage they inflict on the seed crop is hard to estimate as they leave little evidence (Honek & Martinkova 2005; Boierio *et al*. 2010), although one study found Hemipterans to be generalist feeders that exerted low to moderate damage to the seed crop of *Euphorbia* (Boierio *et al.* 2010). Conversely, Lepidoptera and Diptera larave tend to chew their way through the seeds and receptacle, leaving easily recognisable damage (Honek & Martinkova 2005). Furthermore, usually only one caterpillar inhabits a *Celmisia* seed-head, whereas large seed-heads can contain many fly larvae (Molloy 1975). For example, I recorded 11 *Trupanea* from one seed-head in my study. I did not quantify the impact of various seed predators (or all seed predators) on the seed crop of the two *Celmisia* species and their hybrid, but this would certainly provide an interesting avenue for further study.

Many pre-dispersal seed predators experience relatively large rates of parasitoid attacks (Crawley 1992; Sarfati *et al*. 2010), and parasitoids can affect the damage done by seed predators in a seed-head (Swope & Satterthwaite 2012). While I found low levels of Hymenoptera parasitation inside all three *Celmisia* taxa in this study I do not know which insect order they were parasitizing, nor do I know what effect these parasitoids may have on seed-predation rates in *Celmisia*.

Larger inflorescences are thought to be more attractive to pre-dispersal seed predators (Fenner & Thompson 2005), and this has previously been found in other Asteraceae (Fenner *et al*. 2002). There is also evidence for taller plants having higher numbers of seed predators (Hainsworth *et al*. 1984). Boierio *et al*. (2010) found that two *Euphorbia* species growing sympatrically had similar numbers of seed predators to each other and suggested this could be related to the similar fruiting phenologies displayed by their plants. In the *Celmisia* I studied, seeds were collected from all species at the same time; therefore, perhaps the similar numbers of seed predators relates to the shared fruiting phenologies displayed by these *Celmisia* species.

Interestingly, *C. lyallii* is both taller and has larger capitula than *C. spectabilis* (Chapter 3) perhaps providing an explanation for why this species has higher numbers of seed predators than *C. spectabilis*. Furthermore, one of the shortest and smallest of all *Celmisia*, *C. sessiliflora* (Fenner, Lee & Pinn 2001), is recorded as having no seed predators (Spence 1990). While Cumming, Alexander & Snow (1999) found higher levels of pre-dispersal seed predation in hybrid sunflowers, Campbell *et al.* (2002) found *Ipomopsis* hybrids to have intermediate levels or fewer seed predators than their parent species however, Campbell *et al*. (2002) also stress that other factors influence hybrid fitness and these factors should be studied too.

Germination of hybrid seeds

Celmisia germination speed in my study was slower than that reported by Metcalf (1993) and Scott (1975) who found *Celmisia* germinated readily within about four to six weeks. The progeny of reciprocal hybrid crosses can perform quite differently, leading to highly asymmetric postzygotic reproductive isolating barriers (Martin & Willis 2007). This is not likely to be the case in *Celmisia* as Given (1969) describes the hybrids as being the same morphologically regardless of which species was the maternal parent.

Later generation hybrids are thought to be less fit than their parents (Johansen-Morris & Latta 2006), as recombination can rearrange gene complexes that were contributing to hybrid vigour in earlier generations (Rieseberg 1997; Hereford 2009). If plants are capable of self-pollination or clonal reproduction, some hybrids can retain heterosis for longer as recombination is limited via selfing (Lowe & Abbot 2004; Rieseberg & Willis 2007). Fenster & Galloway (2000) found first generation (F_1) hybrids outperformed their parents, but F_3 generation hybrids showed evidence of disrupted gene interactions. Similarly, the later life history stages can also display higher levels of hybrid breakdown than earlier stages. For example Gow, Peichel & Taylor (2007) sampled sticklebacks genetics and found fewer hybrids over successive life history stages, and Nosrati, Price & Wilcock (2011) found that while *Fragaria* hybrids germinated as well as their parents, they produced sterile pollen.

Most studies do not sample beyond the F_1 generation, therefore the failure of later generation hybrids is not detected (Campbell & Waser 2007). Despite this, extrinsic postzygotic reproductive isolation is generally considered to be rare (Lowry *et al.* 2008). For example, Ramsey, Bradshaw & Schemske (2003) found hybrids germinated less well than either parent, but all plants survived to flower, moreover there were no reductions in the fitness of later generation hybrids. Ackerman, Achatz & Weigend (2008) conducted hand crosses and found that most seeds germinated and developed normally and concluded that there were no postzygotic isolating barriers in the *Caiophora* species. Similarly, Dell'Olivio *et al* (2011) found very little postzygotic isolation in *Petunia* species. Another study found F_1

hybrids were fitter than their parents, and, although later generations lost some of this fitness, it was not enough to suggest postzygotic isolation was occurring (Kirk, Vrieling & Klinkhamer 2005).

Kay (2006) found high hybrid fertility in her glasshouse study of *Costus*, however she also noted that hybrids may not be as fit under natural conditions where various environmental factors could limit hybrid success, and they may be less attractive to pollinators. As my seeds were also grown under standard conditions in a glasshouse, any hybrid failure would probably indicate intrinsic isolation as I could not test for environmental effects (Hatfield & Schluter 1999). In the future it would be best to conduct *Celmisia* seed germination trails across a range of environments in which either the hybrids, parents, or both are found (Campbell *et al*. 2008). It has been suggested that the longevity of New Zealand plants may increase hybridisation as short lived species should be disadvantaged by the production of hybrids (Webb & Druce 1984). *Celmisia* are certainly long lived plants, and although I am unsure of what hybrid generation I had, I found no evidence that seed germination in *Celmisia* hybrids was any less successful than their parent species.

Conclusions

Hybrid sterility could be described as the last 'defence' against hybridisation (Schemske 2000), therefore, if none is found, can we assume that reproductive isolation is weak? Although hybrid genotypes are often very variable (Aldridge & Campbell 2007), and hybrid success is environment-dependent (Campbell *et al.* 1998; Arnold 2006), hybrids are a natural feature of angiosperm diversity and their existence is crucial for macro evolution (Campbell & Waser 2007).

Although not abundant in the field, *C. x pseudolyallii* shows no obvious fitness disadvantage; neither the number of seed predators nor seedling germination indicated that this hybrid is less successful than its parent species. The other hybrid combinations, *C. lyallii* X *C. sessiliflora* and *C. viscosa* X *C.* sp*.,* showed the same or better levels of germination than their respective parents, reinforcing the idea that postzygotic isolation is weak in *Celmisia*.

A limitation of this study is that I do not know the generation of hybrids I collected seeds from. Although the hybrids with known parent species were morphological intermediates, it is possible that they were the result of backcrossing events with either parent species or were later generation hybrids. In most cases, putative hybrids assessed by morphological features have later been confirmed as hybrids with molecular studies (Whitney *et al*. 2010). Despite this, I have shown that the *Celmisia* hybrids in my study are performing at least as well as the parent species, thus there is no evidence of postzygotic isolation in the barriers I examined.

CHAPTER FIVE

Synthesis

Flower beetle on *Celmisia lyallii*

Despite the abundance of *Celmisia* in New Zealand's alpine ecosystems this is the first study to quantitatively assess potential reproductive isolating barriers, although a previous study had discussed them (Given 1968). Furthermore, this study provides an experimental test to the floral features postulated by Fenner, Lee & Pinn (2001) to be important in the attraction of floral visitors to *Celmisia*. Many studies of plant hybridisation are not quantitative (Marques *et al.* 2012), and usually only involve a small group of potentially hybridising species. Although I did not find any single reproductive isolating barrier was strong enough to prevent hybridisation in *Celmisia* on its own, I have explained some aspects of reproductive isolation in *Celmisia*. My study has provided important information on the ecology of *Celmisia* through the examination of four potential reproductive isolating barriers in a natural community.

It is likely that geographic isolation of *Celmisia* species contributes to their lack of hybridisation, as it is an important barrier in other plant species (Husband & Schmeske 2000; Ramsey, Bradshaw & Schemske 2003; Lowry *et al.* 2008); although levels of sympatry in *Celmisia* are high, not all 65 *Celmisia* species native to New Zealand are found together. Geographic isolation could be viewed as the first filter in a series of sieves that work together to prevent hybridisation in *Celmisia* (Figure 5.1). My thesis illustrates the idea of various barriers filtering out hybrids, as fewer and fewer *Celmisia* species pairs were used with each new reproductive isolating barrier I examined (Table 5.1).

I applied a novel approach to test for differences in flowering phenology (Chapter Two) in a group of potentially hybridising plants, and surprisingly (despite the constraints of a short flowering season) found some evidence for segregation of flowering in alpine plants. I have found evidence (Chapter Three) to support the recent work by Campbell *et al*. (2010; 2012) who asserted that the New Zealand alpine pollinating fauna is not as generalist as previously thought. It is particularly interesting that the insects in my study showed preferences within *Celmisia* given that the morphological differences between species were so slight (Table 5.1). I found evidence to suggest that *Celmisia* hybrids produce seeds that are at least as fertile as their parent species and harbour similar number of seed predators as their parent species (Chapter Four).

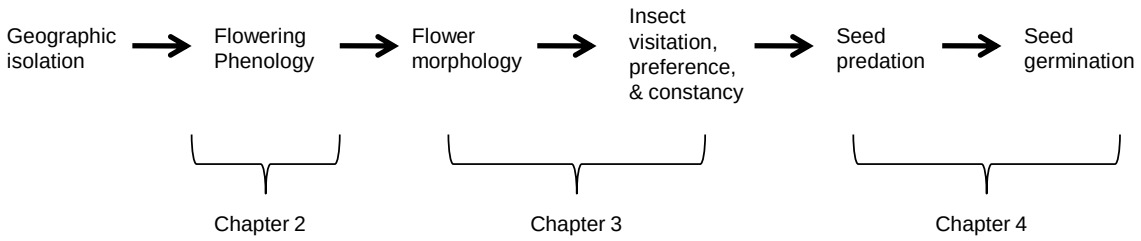

Figure 5.1: Flow chart of potential reproductive isolating barriers in *Celmisia* with a breakdown of where each chapter fits into the process.

Table 5.1: Presence or absence of multiple potential reproductive isolating barriers in *Celmisia* across several *Celmisia* species pairs examined in my thesis. Blank spaces indicate that a barrier was not examined for the respective species pairs.

Celmisia species pairs	Natural hybrids reported[ℓ]	Flowering overlap*	Morphological Differences[≠]				Insect preferences[+]					Higher hybrid seed predation	Lower hybrid seed germination
			overall	height	size	ratio	Overall	Colletidae	Empididae	Syrphidae	Tachinidae		
ANGDIS	N	0.365	N	N	N	Y	Y	Y	N	N	N		
ANGGRA	N	0.000	N	Y	Y	Y	Y	N	Y	N	Y		
ANGVIS	N	0.064	N	Y	Y	Y	Y	N	N	Y	N		
GRASPE	Y	0.028	N	Y	Y	Y	Y	Y	N	N	N		
LYASPE	Y	0.234	N	Y	Y	Y	N	N	N	Y	N	N	N
LYASES	Y	0.052	Y	Y	Y	Y							N
SESSPE	Y	0.313	Y	Y	Y	Y	Y	Y	Y	Y	Y		

ANG = *C. angustifolia*, DIS = *C. discolor*, GRA = *C. graminifolia*, LYA = *C. lyallii*, SES = *C. sessiliflora*, SPE = *C. spectabilis*, VIS = *C. viscosa*
N = no, Y = yes
[ℓ]Natural hybrids defined in Chapter Two
*measured using Schoener formula (Chapter Two); where 0 = no overlap between two species and 1 = complete overlap between two species.
[≠]overall = were the species pairs different across all the floral traits measured?, height = scape height, size = capitulum diameter, ratio = ratio of ray (white coloured) to disc (yellow coloured) florets (Chapter Three)
[+]proportion of insect visits to one *Celmisia* species ≥ 0.50 (Chapter Three)

Coyne & Orr (2004) suggest that studies of reproductive isolation quantify the strength and importance of each reproductive isolating barrier in relation to other barriers and total reproductive isolation. Several studies have utilised this approach with their study species (Ramsey, Bradshaw & Schemske 2003; Kay 2006; Martin & Willis 2007), and one review paper has calculated measures of reproductive isolation from a range of other studies (Lowry *et a*l. 2008). I chose not to apply this method to the reproductive isolating barriers studied in *Celmisia*. Firstly, because I have multiple *Celmisia* species pairs and often different pairs were used to assess the various barriers, I therefore cannot calculate the strength of barriers across all the species in this study. Secondly, I believe that some of the measures of reproductive isolating barrier strength are not an appropriate measure of reproductive isolation. For example, studies that only examine one aspect of pollinator behaviour (such as constancy: Ramsey, Bradshaw & Schemske 2003; Kay 2006), may not get the full picture of pollinator-mediated reproductive isolation (e.g. insect preference). Lastly, the methods I employed of quantifying reproductive isolation do not align with the methodology of Ramsey, Bradshaw & Schemske (2003). For example, I used a null model approach to determine whether the overlaps I had observed between *Celmisia* species were different from random. This approach told me more about the role of reproductive isolation in *Celmisia* than if I had based my inferences on the overlap values alone. Furthermore, measures of reproductive isolation usually do not consider the point in a plant's life history that the respective barriers act, meaning measures of prezygotic isolating barriers are likely to be underestimated as prezygotic barriers are thought to contribute more to reproductive isolation by acting earlier in an organism's life history (Lowry *et al*. 2008).

Most plants are reproductively isolated by a suite of barriers that accumulate across every life history stage (Rieseberg & Willis 2007; Yang, Gituru, & Guo 2007); it appears that this may occur in *Celmisia* too, with no single barrier in my study being strong enough to prevent hybridisation on its own. I did however find evidence to suggest prezygotic isolating barriers played a greater role in the reproductive isolation of *Celmisia* than postzygotic barriers, a finding that is consistent with the majority of previous studies (Lowry *et al*. 2008). In *Celmisia*, the reproductive isolating barriers I studied are far from complete, and the natural hybrids I found and studied at Craigieburn Valley Ski Area appear to be fertile and persisting in the environment.

Suggestions for future work

While I found some evidence for two prezygotic reproductive isolating barriers in *Celmisia* (flowering phenology and insect floral preference), neither of these two factors are strong enough on their own to explain the reported lack of wild hybrids. There is an opportunity for future work in this system. Although investigating every individual potential reproductive isolating barrier is a tedious prospect, it is the most useful way to study speciation (Lowry *et al*. 2008). Some aspects of reproductive isolation are perhaps more interesting to study in *Celmisia* and I list these below:

1. Develop a molecular phylogeny for *Celmisia* species.
    a. Compare *Celmisia* geographic distributions to the phylogeny to see if closely related species are allopatric (as suggested by Given 1968).

b. Use the phylogeny to undertake a detailed study of flowering phenology.

2. Armstrong (2003) found differences in microhabitats prevented hybrids being successful in alpine Australian *Ranunculus*. A similar study could provide more information about how many *Celmisia* can live in sympatry.

3. Determine whether *Celmisia* can self-pollinate; there is a lack of information on rates of self-pollination in New Zealand plants (Webb & Kelly 1993; Newstrom & Robertson 2005) in general. Moreover, self-pollination can be a good method of reproductive isolation in plants (Wendt *et al*. 2002; Lowe & Abbott 2004; Martin & Willis 2007). As *Celmisia* suffer high levels of seed predation in the field, a breeding system study would need to take place in a glasshouse or involve insecticides in the field to avoid loss of seeds to insects.

4. Determine whether post-pollination but prezygotic isolating barriers are effective in *Celmisia* with glasshouse hand crossing trials (to avoid seed predation). Anecdotal evidence (Chapter 1) would suggest this is not the case.

5. Running a *Celmisia* germination experiment in a natural environment would determine whether extrinsic postzygotic reproductive isolating barriers affect hybrid survival. This would be best in species that have differences in underlying habitat, or across *Celmisia* species pairs with and without previous records of hybridisation. Future studies of *Celmisia* hybrids would benefit from knowing what generation of hybrids are present in the field (Milne & Abbott 2008).

Does hybridisation matter?

The loudest critics of the Biological Species Concept (BSC) are botanists (Ramsey, Bradshaw & Schemske 2003), who have long noted the preponderance of plant hybrids across a variety of plant families (Ellstrand, Whitkus & Rieseberg 1996; Whitney *et al*. 2010). We should stop viewing speciation as an endpoint in the evolution of species because reproductive isolation is rarely complete (Hendry *et al*. 2007). Furthermore, plants are generally flexible in their reproductive isolating barriers, with various barriers becoming weaker or stronger with changes in the environment (Wendt *et al.* 2002).

It is increasingly becoming clear that hybridisation has provided an important starting point for the evolution of new species (Herschl & Roy 2007), with Mallet (2007) describing them as "hopeful monsters" with new combinations of genes that could become adaptive in the right situation. Furthermore, it appears that some genera are capable of maintaining multiple species in sympatry despite being susceptible to hybridisation (Milne *et al*. 1999), and reproductive isolation does not need to be absolute in order for species to develop genetic differentiation (Rieseberg & Willis 2007). Additionally, it is particularly likely that species that have undergone recent speciation events, such as

the New Zealand alpine flora, will have lower levels of reproductive isolation (Rieseberg & Willis 2007).

Previous studies have demonstrated that plants are highly variable when it comes to reproductive isolation, therefore making generalisations difficult (Ramsey, Bradshaw & Schemske 2003; Aldridge & Campbell 2007). Even within *Celmisia* there is a huge amount of variation in reproductive isolation across various species pairs (Table 5.1).

Conclusions

A recent comparison of hybridisation in the New Zealand flora with other similarly sized floras concluded that New Zealand does not have larger numbers of hybrids as previously thought (Wilson & Lee 2012). What the New Zealand flora does have is a large number of genera that have undergone recent speciation events (Abbott, Ritchie & Hollingsworth 2008; Linder 2008), and therefore a suite of species that are useful in studies of factors contributing to reproductive isolation. I found *Celmisia* to be weakly isolated by both differences in flowering time and flower visitor preferences, and not isolated by the two postzygotic barriers I examined.

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Appendix 1

Introduction to the *Celmisia* species from Craigieburn Valley Ski Area

All scale lines indicate a length of 5 mm.

Celmisia angustifolia

Celmisia discolor

Celmisia glandulosa

Celmisia graminifolia

Celmisia haastii

Celmisia laricifolia

Celmisia lyallii

Celmisia sessiliflora

Celmisia spectabilis

Celmisia verbascifolia

Celmisia viscosa

Celmisia walkeri

Celmisia x pseudolyallii

Appendix 2

List of flower visitors to *Celmisia*

Table A2.1: List of flower visitors observed during the course of this study. Flower visitors that were not seen at an array were observed elsewhere. Note the record of one bird, *Zosterops lateralis*.

Order	Family	Genus & species	Observed at arrays
Coleoptera	Curculionidae		N
	Melyridae	*Dasytes Sp*	N
Diptera	Acroceridae	*Helle sp.*	N
	Empididae	*Hilaria sp.1*	Y
		Hilaria sp.2	Y
	Sryphidae	*Allograpta sp.*	Y
		Helophilus sp.	Y
		Melangyna novaezelandiae	Y
		Merodon sp.	Y
		Platycherirus Sp.	N
	Stratiomyidae	*Beris sp.*	N
		Odontomyia Sp.	Y
	Tabanidae	*Scaptia adrel*	Y
	Tachinidae	*Protohystricia sp.1*	Y
		Protohystricia sp.2	Y
		Unknown 1	Y
		Unknown 2	Y
		Unknown 3	Y
	Tephritidae	*Trupanea*	N
	Unknown	Unknown	Y
Hemiptera	Lygaeidae	*Rhypodes*	N
Hymenoptera	Colletidae	*Hylaeus Sp.*	Y
	Colletidae	*Leioproctus sp.*	Y
	Colletidae	*Leioproctus vestitus*	Y
	Halictidae	*Lassioglossum sp.*	Y
Lepidoptera	Lycaenidae	*Lycaena salustius*	Y
	Nymphalidae	*Argyrophenga antipodum*	N
Orthoptera	Acrididae	*Phaulacridium marginate*	Y
Thysanoptera	Unknown	Unknown	Y
Passeriformes	Zosteropidae	*Zosterops lateralis*	N

Appendix 3

Additional information for model selection using an Information Theoretic approach

Table A3.1: Base set of candidate models for testing whether insect preference is driven by floral characteristics. Position = capitula on the edge or interior of the array, height = scape height, ratio= ray/disc (white/yellow) ratio, size = capitulum diameter, sex = phenological flowering stage of the capitulum (1, 2, 3, or 4), order = insect order.

Model	Response	Predictors
1	First Species	random factor
2	First Species	random factor + position
3	First Species	random factor + height
4	First Species	random factor + size
5	First Species	random factor + ratio
6	First Species	random factor + sex
7	First Species	random factor + order
8	First Species	random factor + position + height + ratio + sex + order
9	First Species	random factor + position + height + size + sex + order
10	First Species	random factor + height + ratio
11	First Species	random factor + height + size
12	First Species	random factor + height + sex
13	First Species	random factor + height + order
14	First Species	random factor + size + sex
15	First Species	random factor + size + order
16	First Species	random factor + size + sex + order
17	First Species	random factor + height + sex + order

Explanation of why some models were left out for some of the arrays

For various reasons not all candidate models were used for each array type. I have explained why for each *Celmisia* pair-wise array below.

C. angustifolia: C. discolor
All models were used.

C. angustifolia: C. graminifolia
All models were used.

C. angustifolia: C. viscosa
Low replication lead to models 9 and 15 failing to converge, therefore I removed these models from the candidate set.

C. graminifolia: C. spectabilis
Some models including the variable height failed to converge (these were: 3,8,9,10,11,12,&13). I removed height from models 8 and 9, so I could still include a global model in the candidate set.

C. lyallii: C. spectabilis
Low replication probably lead to model 11 failing to converge, therefore I removed this model from the candidate set.

C. sessiliflora: C. spectabilis
Due to slight collinearity between the height and size variables (Figure A3.1) in this data set the two models that contained both for these parameters were excluded from the candidate set.

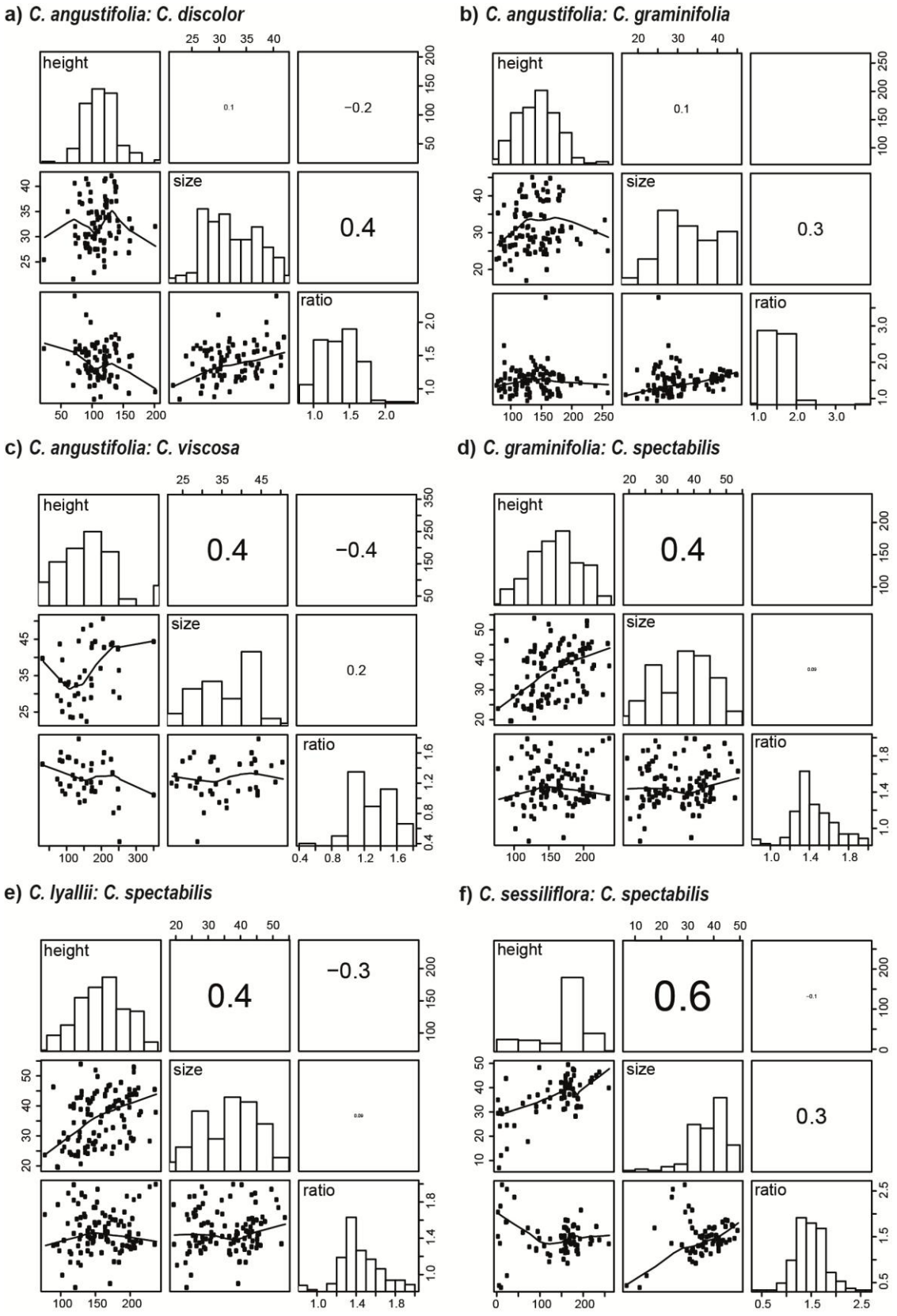

Figure A3.1: Pair plots for assessing whether potential parameters are collinear. a)-e) show no indication of collinearity between any of the potential predictors, but f) shows slight collinearity between height and size (pair-wise correlation value >0.5) . The figure was drawn using the R package 'AED' (Zuur 2010), in R version 2.14.2 (R Core Development Team 2012).

Estimates and Unconditional standard errors for each *Celmisia* array
All values included in these tables are still logit linked.

C. angustifolia: C. discolor

Table A3.2: Estimates and unconditional standard errors from the best model only.

Parameter	Estimate	Std. Error
Intercept	4.885	1.737
Scape height	0.054	0.012
Ray/disc ratio	1.172	-6.382

C. angustifolia: C. graminifolia

Table A3.3: Estimates and unconditional standard errors from the best model only. -

Parameter	Estimate	Std. Error
Intercept	2.736e+01	2.018e+03
Capitulum diameter	-6.057e-01	1.153e-01
sex - 2	-1.799e+01	2.018e+03
sex - 3	-1.638e+01	2.018e+03
sex - 4	-1.382e+01	2.018e+03
order - Hymenoptera	-9.342e-02	5.734e-01
Position - outside	-6.748e-01	6.122e-01
Scape height	3.515e-02	8.149e-03

C. angustifolia: C. viscosa

Table A3.4: Estimates and unconditional standard errors from the best model only.

Parameter	Estimate	Std. Error
Intercept	-37.770	13.868
Capitulum diameter	0.861	0.026
Scape height	0.072	0.026

C. graminifolia: C. spectabilis

Table A3.5: Model averaged estimates and unconditional standard errors

Parameter	Estimate	Std. Error
Intercept	-4.824e+01	4.463e+3
Capitulum diameter	1.067e+00	4.179e+00
sex - 2	1.692e+01	4.463e+03
sex - 3	8.334e+00	4.463e+03
sex - 4	1.234e+01	4.463e+03
order - Hymenoptera	9.706e-01	9.784e-01
Position - outside	3.578e-02	1.218e+00
Scape height	-3.974e-02	1.331e-02
Ray/disc ratio	-6.524e-03	6.748e-01

C. lyallii: C. spectabilis

Table A3.6: Model averaged estimates and unconditional standard errors

Parameter	Estimate	Std. Error
Intercept	-5.206e+00	3.181e+03
Capitulum diameter	-1.666e-01	3.054e-02
sex – 2	1.652e+01	3.637e+03
sex – 3	1.750e+01	3.637e+03
sex – 4	1.467e+01	3.637e+03
order - Hymenoptera	1.247e+00	1.121e+00
order – Lepidoptera	1.728e+01	9.476e+03
Position – outside	-2.884e-01	7.953e-01
Scape height	-3.265e-02	5.464e-03
Ray/disc ratio	8.625e-01	1.142e+00

C. sessiliflora: C. spectabilis

Table A3.7: Model averaged estimates and unconditional standard errors

Parameter	Estimate	Std. Error
Intercept	-4.298e+00	2.723e+03
Capitulum diameter	-1.666e-01	3.054e-02
sex - 2	1.629e+01	3.215e+03
sex - 3	1.725e+01	3.215e+03
sex - 4	1.444e+01	3.215e+03
order - Hymenoptera	1.128e+00	1.117e+00
order - Lepidoptera	1.663e+01	7.684e+03
Position - outside	-2.884e-01	7.953e-01
Scape height	-3.244e-02	5.403e-03
Ray/disc ratio	8.625e-01	1.142e+00

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Appendix 4

Herbarium voucher specimens

CELang	J.L. Gosden 042277 (CANU)
CELdis	J.L. Gosden 042275 (CANU)
CELgla	J.L. Gosden 042274 (CANU)
CELgra	J.L. Gosden 042271 (CANU)
CELhaa	J.L. Gosden 042269 (CANU)
CELlar	J.L. Gosden 042267 (CANU)
CELlya	J.L. Gosden 042265 (CANU)
CELses	J.L. Gosden 042263 (CANU)
CELspe	J.L. Gosden 042262 (CANU)
CELver	J.L. Gosden 042259 (CANU)
CELvis	J.L. Gosden 042258 (CANU)
CELwal	J.L. Gosden 042255 (CANU)
CELlxs	J.L. Gosden 042251 (CANU)
CELvis hybrid	J.L. Gosden 042250 (CANU)